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The inability of wt-Huningtin expressed in C. elegans indicates that its anti-apoptotic function affects non-conserved aspects of Caspase-9 activation.

We established a cellular model for the apoptotic signaling of $p75^{NTR}$. The conditionally immortalized neuronal cells used is the first system in which $p75^{NTR}$ induces apoptosis in the absence of any co-stimuli. The tools are now in hand to identify the intracellular signaling components involved in transmitting the apoptotic signal from this receptor. A close homologue of $p75^{NTR}$, NRADD, was also characterized. Despite its close homology, NRADD signals apoptosis by a different mechanism. Its activity is controlled by N-glycosylation and dominant negative constructs interfere with apoptotic stimuli generated at the endoplasmatic reticulum.

Transgenic expression of the viral PAN-death receptor inhibitor E8 protected mice from excitatory damage induced by kainic acid. This observation is highly relevant for neurodegenerative diseases like multiple sclerosis where excitatory and immune mediated damage is observed.

New insight into the assembly of death domain complexes was obtained by analyzing published structures of death domains heterodimeres. It emerges that death domains can interact in such a way to mediate multivalent interactions and not only monovalent associations as currently thought.

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## Table of Contents

## Introduction

This report is a final addendum to the report submitted last year (6/2000). Funding for this fifth year was obtained through a no cost extension agreement of the original grant. For a detailed description of the progress during the regularly funded 4 year period the reader is referred to last years final report submitted on June 2000. Here only a listing of the key accomplishments and the publications generated during the whole period are presented. The focus of this report is the results obtained during this last year funded through the no cost extension.

Most of the progress discussed in this report pertain the revised SOW task #2 (neuronal apoptosis). We previously established that wtHuntingtin (Htt) confers anti-apoptotic activity in a neuronal cell line. Here we describe attempts to more closely define the mechanism by reconstituting the anti-apoptotic effects of Htt by using recombinant proteins. Because our cellular experiments indicated that the Htt targeted the conserved effector machinery of apoptosis we also investigated the function of Htt in Caenorhabditis elegans.

Towards the goal of identifying the signaling mechanisms of the $p75^{NTR}$ death receptor, we further characterized a neuronal cellular system. This model system will be the basis for the identification of the signaling components that mediate the apoptotic response initiated by $p75^{NTR}$. Furthermore, a close homologue of $p75^{NTR}$, NRADD, was discovered. The pro-apoptotic activity of this new molecule was characterized in several cell lines. Insight into the mechanism of NRADD was obtained through characterization of its post-translational modifications and production of a truncation mutant with dominant negative properties.

To establish a physiological role of death receptors in neuronal systems we continued the characterization of transgenic mice expressing the PAN-death receptor E8. A transgenic mouse line expressing this vFLIP in the CNS was screened for potential CNS effects. Challenging the transgenic mice and their wild type littermates with kainic acid revealed a significant protection from seizures as well as mortality induced by this agent that induces excitatory damage.

**Body:**

**1) In vitro activities of recombinant Htt.**

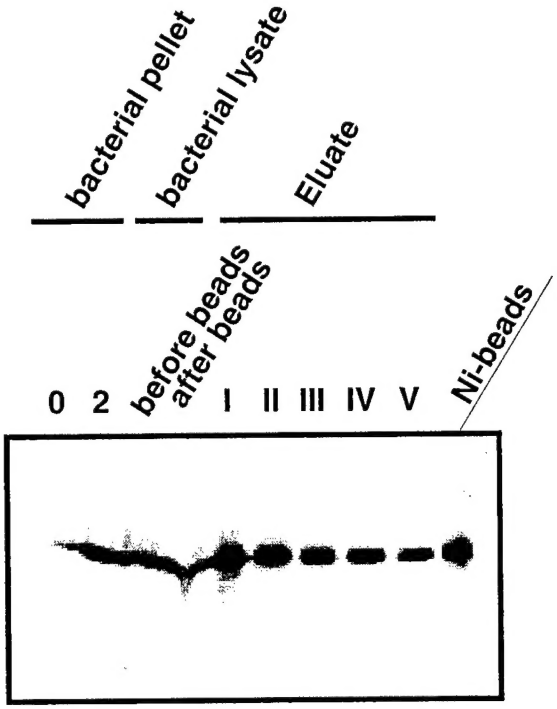
Analysis of conditionally transformed striatal neurons (ST14A) stably expressing Htt had shown that they were protected from a variety of apoptotic insults (Rigamonti et al., 2000). The protection was also observed against pro-apoptotic Bcl-2 members and Caspase-9. This anti-apoptotic activity profile strongly suggested that the target of Htt was the apoptotic effector machinery. Htt did not protect from apoptosis induced by a constitutively active Caspase-3 construct, narrowing potential targets to events surrounding Caspase-9 activation. This hypothesis was further confirmed by the observation that Htt directly bound to the catalytic domain of Caspase-9 when co-expressed in 293 cells.

Because the results were obtained from stable cell lines possible artifacts due to the selection process could occur. Further, the in vivo experiments do not distinguish between direct and indirect effects. Both of these concerns could be eliminated if an in vitro system using only purified components could be developed. In vitro activation systems for Caspase-9 have been described in the literature by several investigators (Cain et al., 2000; Zou et al., 1999). The purified proteins necessary are Caspase-9, cytochrome c, and Apaf-1. The first two are readily available while recombinant Apaf-1 has poor solubility properties making isolation more laborious. To avoid these experimental challenges during the initial experiments Apaf-1 and Caspase-9 were not added to the reaction mixture as a purified protein but as a S-100 cell lysate. This is the same system used by Xiadong Wang that led to the discovery of Apaf-1 and cytochrome c. This approach would reduce the experimental challenge to the production of recombinant Htt.

Full length Htt is a protein of 3144 amino acids presenting a formidable challenge for recombinant expression. However, the cellular experiments had revealed that the protective function of Htt is located within the first N-terminal 548 AA. We therefore attempted to express this fragment as a recombinant protein. This fragment is still substantially longer than the exon-1 Htt- constructs (80 AA) described in the literature (Scherzinger et al., 1997). Already with the shorter exon-1 constructs solubility presents a problem particularly if the poly-glutamine stretch is expanded as it is in the mutated protein. Therefore only constructs expressing wtHtt (1-548) with 23 poly-glutamines were engineered.

Standard PCR techniques were used to generate two fusion constructs. One as a C-terminal $His_6$ fusion protein in the pET30a vector, the other as C-terminal GST fusion protein in the pGEX-4T-1 vector. Only the results with the $His_6$ construct are shown as similar results were obtained with the GST fusion protein. Induction with IPTG yielded a protein of the right molecular weight (~60kd) that was only detectable by western blotting with α-Htt antibodies (Fig. 1). Usually this vector and purification conditions would yield mg amounts of recombinant proteins that are easily detectable by Coomassie staining. The lack of Coomassie stainable bands indicates that only small amounts of the protein were expressed. Analysis of the fractions during purification revealed that the protein was partially soluble and bound to the Ni-beads with only ~50% efficiency. Elution with immidazole (250 mM) was sluggish and incomplete.

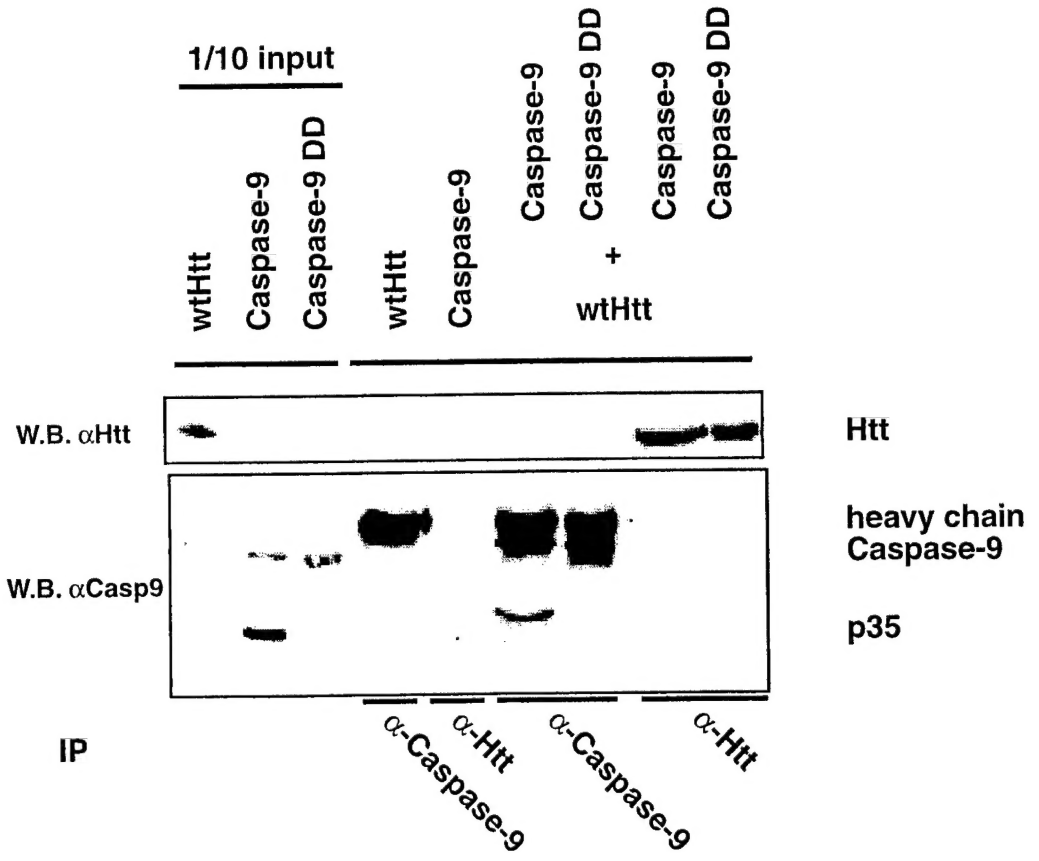
**Fig. 1**




*A S-tag/$His_6$-wtHtt (1-548) fusion protein was produced in E. coli transformed with the bacterial expression construct. The bacterial culture was induced with 1 mM IPTG at $OD_{600}$ of 0.5 for 2 hr at RT. The bacterial pellet was resuspended and lysed by sonication. The cleared lysate was bound to Ni-beads and washed extensively. Elution was achieved by 250 mM immidazole. Aliquots of each fraction were separated on a SDS-PAGE and analyzed by western blot using and α-Htt antibody.*

The amounts of recombinant Htt expressed by the bacteria were too low to perform in vitro Caspase-9 inhibition experiments as most likely stoechiometric amounts are needed. However, to assay whether the recombinant material had any of the properties observed with Htt expressed in mammalian cells co-immunoprecipitation experiments were performed. We have previously shown that Htt and pro-Caspase-9 co-expressed in 293 cells bind to each other. When the analogous experiment is performed with recombinant Caspase-9 and Htt no binding is observed (Fig. 2) A cleavage site mutant form of Caspase-9 was included to mimic the activated but not cleaved form of Caspase-9. Htt did not bind to this form either. To exclude the possibility that binding required additional factors 293 lysates were added. Recombinant Htt still did not interact (not shown).

These results suggest that bacterially expressed Htt is not able to participate in the same molecular interactions as the protein expressed in eukaryotic cells and is probably in a miss folded conformation. In addition the amounts obtained are miniscule. Attempts are under way to use a baculovirus system to produce properly folded protein.




*The indicated recombinant proteins were diluted 1:10 in buffer A and immunoprecipitations were performed a shown. No cross-reactivity of either antibody was observed (lanes 4, 5). No interaction of wtHtt with Caspase-9 could be observed (lanes 6-9).*

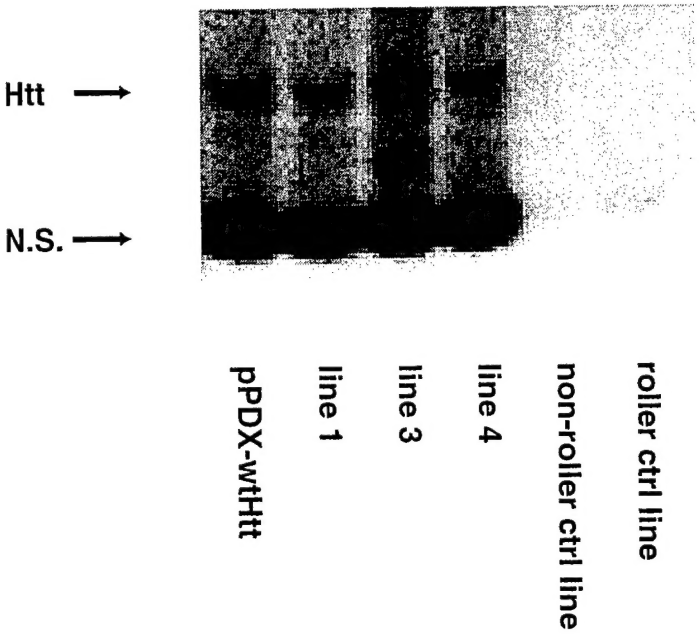
**2) Function of Htt in C. elegans**

The apoptotic effector machinery reveals a high degree of conservation between vertebrates and lower organisms including C. elegans. Because our cellular experiments indicated that Htt was directly affecting this pathway transgenic C. elegans were generated to evaluate whether the Htt target was also evolutionary conserved. The C. elegans genome does not contain a Htt homologue providing a convenient null background.

Wt- and mu-Htt (1-548) were subcloned into a vector that uses the heat shock promoter to drive expression of the transgene. This plasmid was then microinjected into the syncytium of L4 larvae or young adult animals. A plasmid encoding the rol-6 gene was co-injected as a marker to identify the transgenic animals. The rol-6 gene alters the animals movement to a rolling motion that is easily distinguished from the movement of the wild type worms. Roler animals were selected and propagated and the F2 generation

was subjected to PCR analysis (Fig. 3). Clearly, the roller phenotype correlated with the transmission of the transgene.

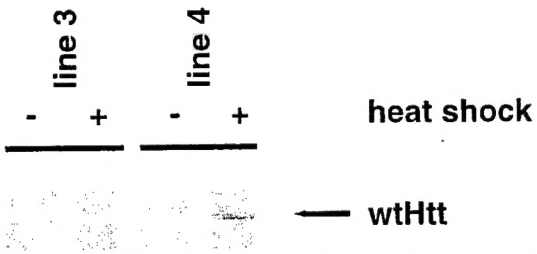
**Fig. 3**




*Single worm PCR analysis was performed on three stable roller/wtHtt lines, a non-roller and a roller control line. Vector pPDX-wtHtt was used as a positive control. Single worms were lysed in 5 µl lysis buffer (10 mM Tris HCl, pH8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin), supplemented with 10mg/ml Proteinase K, by freezing in liquid $N_2$ for 20 min, followed by incubation at 60 °C for 2 hr and heat inactivation of the proteinase. Aliquots were then subjected to PCR using KlenTaq Advantage polymerase (Clonethech). N.S. non specific band*

To directly document protein expression worms were heat shocked and the protein extract was analyzed on a western blot with αHtt antibodies (Fig. 4). An induction of the right size Htt immunoreactive band was observed upon heat shock.

**Fig. 4**




*A petri dish with ~ 500 animals was heat shocked where indicated at 30 °C for 1 hr and the worms collected , washed in PBS, resuspended in Buffer A and flash frozen in liquid $N_2$. Frozen animals were ground to a fine powder in a pre-cooled mortar and centrifuged at 10,0000 x g for 10 min. Equal amount of protein was loaded on the SDS-PAGE gel and analyzed by αHtt western.*

To assess the functional consequences of Htt expression in C. elegans the number of pharyngeal cells was compared between heat shocked, non heat shocked, and roler controls. Of the 131 programmed cell deaths observed during C. elegans development 22 occur in the pharynx. This represents a convenient area for easily assaying the effects of the Htt transgene. Adult animals were heat shocked for one hr and allowed to lay eggs for another hr to induce Htt expression in the developing embryo. The resulting offspring was scored in the L3 stage by Normaski microscopy for changes in the total number of pharyngeal cells. No statistical significant differences were observed between wt Htt and wild type control worms (not shown).

To improve the sensitivity of the assay Htt worms were bred into different genetic backgrounds. For a schematic of the breeding protocol see Fig. 5.

The CED-3 (n2877) mutation is a weak loss of function mutation that only partially suppresses apoptosis, which results in seven additional cells in the pharynx. While a weak Htt effect might go unnoticed in the N2 background, in the CED-3 background a more pronounced difference is expected. The animals were analyzed in the L3 stage to score for pharyngeal cells. At this stage these cells are not yet obstructed by muscle tissue. The same number of pharyngeal cells was observed in the heat shocked (34.96 ±4.1) as in the non-heat shocked controls (39.2 ±5). This indicates that expression of wtHtt in C. elegans does not affect developmental cell death.

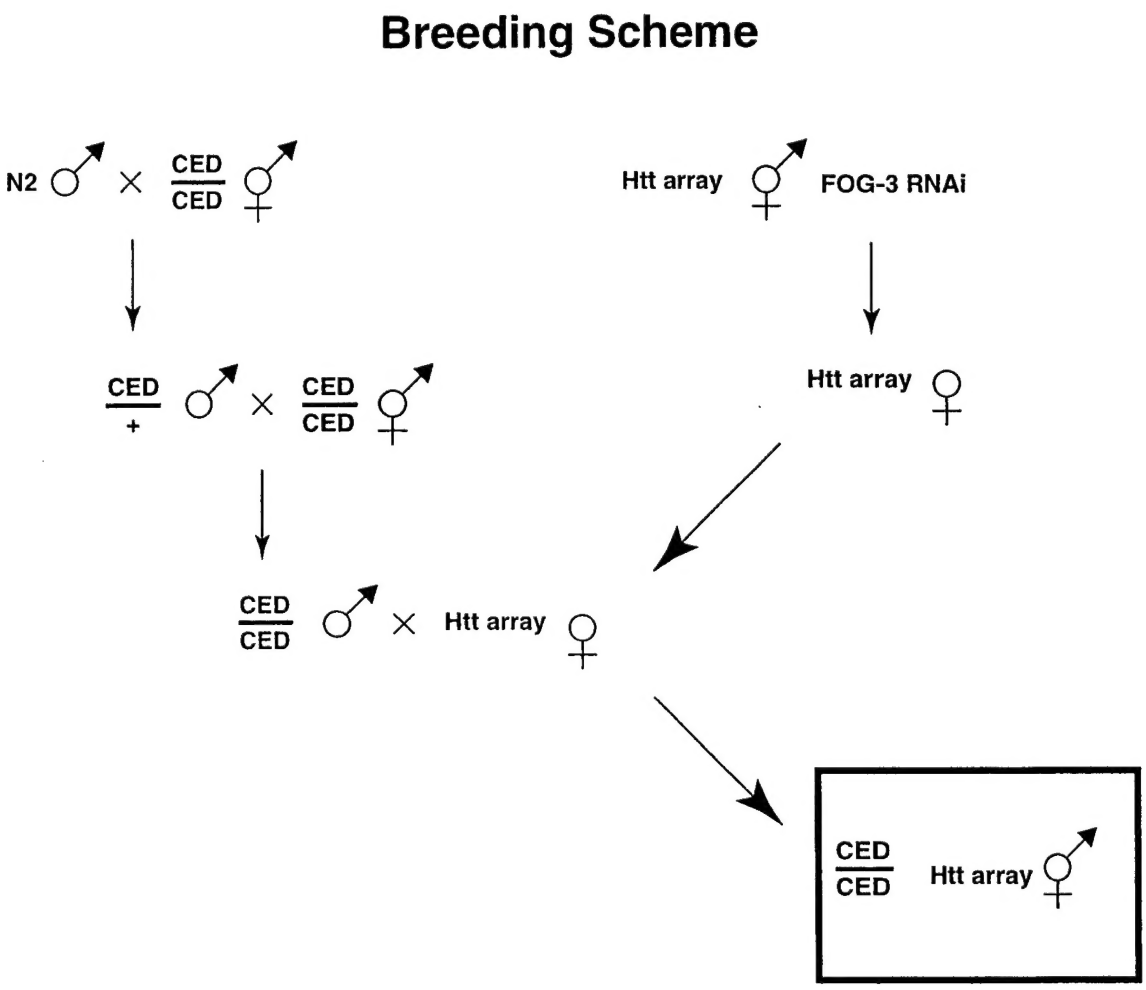
To increase the detection sensitivity for apoptotic bodies the CED-1 mutant was used. The CED-1 mutants (n1995) do not have a defect in the apoptotic effector machinery but in the clearance of dead cells. Apoptotic corpses persist much longer in the CED-1 versus wild type making it easier to obtain full count of all dying cells. Animals are scored early in L1 for the appearance of cells with a button like appearance. Counting of wt Htt/CED-1 and vector/CED-1 revealed no difference in the number of corpses observed (14.58 ±3.9, n=65 wtHtt, 14.43 ±3.5, n=55 vector). These data confirm the observations in the wt type and CED-3 backgrounds, corroborating the lack of an anti-apoptotic function of Htt in the nematode.

Caspase-9 activation in vertebrates and CED-3 activation in the nematode is different as the former requires at least Apaf-1and cytochrome c the latter only needs

CED-4. Therefore these results suggest that wtHtt interferes with the activation process for Caspase-9. This is unexpected as Htt binds to the catalytic domain of Caspase-9 and CED-3 (not shown). However, given the size of Htt one can easily envisage steric hindrances that do not permit the proper assembly of a Caspase-9 Htt complex into functional apaptosome.

We are continuing to explore the function of the Htt in C. elegans by analyzing the muHtt worms that were made in parallel.

**Fig. 5**




*In order to bread the extrachromosomal Htt array into different genetic background, Htt females were generated by FOG-3 RNAi. The resulting females are bred with homozygous males carrying the desired CED mutation. The males are generated by crossing N2 males with CED hermaphrodites, followed by backcross of the resulting CED heterozygote males with CED hermaphrodites.*

**3) A cellular system for the apoptotic signaling of $p75^{NTR}$.**

This part of the project has been submitted for publication to the J. Biol. Chem.. We have addressed the reviewers comments and are awaiting the decision from the editiorial office. Included here is the summary of the paper and appended is the full manuscript.

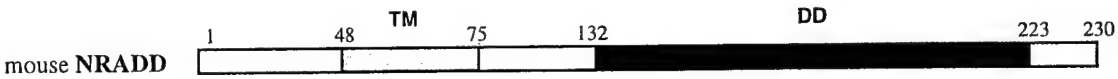

**Summary**

The p75 neurotrophin receptor ($p75^{NTR}$) belongs to the tumor necrosis factor TNFR/NGFR receptor superfamily. In some cells derived from neuronal tissues it causes cell death through a poorly characterized pathway. We developed a neuronal system using conditionally immortalized striatal neurons, in which the expression of $p75^{NTR}$ is inducibly controlled by the ecdysone receptor. In these cells $p75^{NTR}$ induces apoptosis through its death domain (DD) in a neurotrophin growth factor (NGF) independent manner. Caspases 9, 6, 3 are activated by receptor expression indicating the activation of the common effector pathway of apoptosis. Cell death is blocked by a dominant negative form of Caspase 9 and Bcl-$X_L$ consistent with a pathway that involves mitochondria. Significantly, the vFLIP E8 protects from $p75^{NTR}$ induced cell death indicating that death effector domains are involved. A $p75^{NTR}$ construct with a deleted DD dominantly interferes with $p75^{NTR}$ signaling, implying that receptor multimerization is required. However, in contrast to the other receptors of the family, $p75^{NTR}$ mediated apoptosis does not involve the adaptor proteins FADD or TRADD, and the apical Caspase 8 is not activated. We conclude that $p75^{NTR}$ signals apoptosis by similar mechanisms as other death receptors but uses different adaptors and apical caspases.

**4) Characterization of NRADD, a p75$^{NTR}$ homologue.**

In the previous reports the initial discovery of NRADD was already described. This p75$^{NTR}$ homologue was found by searching the EST databank for death domain containing proteins. Cloning of the full length protein revealed that it not only contained a death domain that is highly homologous to p75$^{NTR}$ but the remainder of the putative intracellular and transmembrane domains are very homologous too. However the putative extracellular domain is very short (48 AA) and has no homology to p75$^{NTR}$ or any other members of the TNFR superfamily. Expression of NRADD induces cell death in some neuronally derived cell lines but not in other cells tested. During the current reporting period we have characterized NRADD further with the emphasis on molecular dissection of the mechanism and cellular experiments aimed at elucidating the physiological role of NRADD.

**Fig. 6**

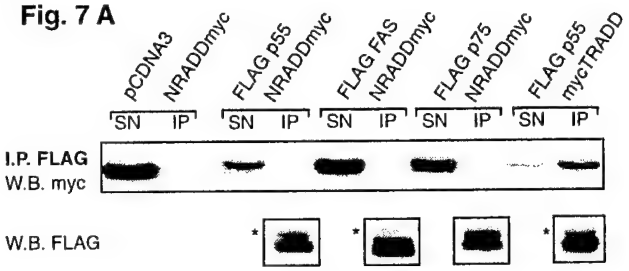



*Cartoon outlining the domains of NRADD. TM: trans membrane, DD: death domain.*

The death domain functions as a homotypic interaction domain. To reveal any interaction partners co-immunoprecipitation experiments were performed. The prototypic death receptors FAS and p55 (TNFR1) as well as p75$^{NTR}$ were assayed (Fig. 7 A). Immunoprecipitation of the FLAG tagged receptors and western blotting with α-myc to detect NRADD revealed no binding to any of the molecules tested. The positive control, binding of TRADD to FLAGp55, yielded a strong mycTRADD co-IP band. The analogous experiment with FADD and RAIDD also failed to show any interactions with NRADD (not shown).

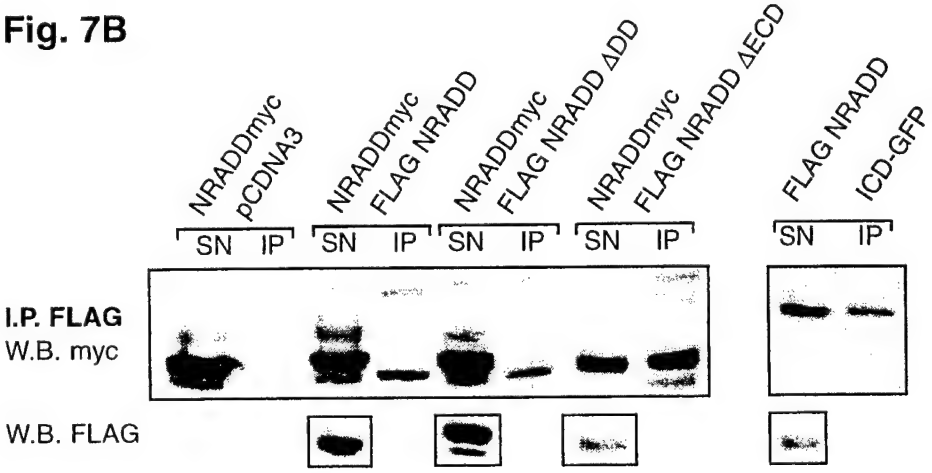
Several members of the death receptor family have been shown to homo-oligomerize (Siegel et al., 2000). The potential for NRADD to form homo-oligomeres was therefore assayed in the 293 system (Fig. 7 B). Significant interaction between the full length NRADD molecules was only found for a minor form NRADD that migrated on the SDS-PAGE gel ahead of the predominant band. Mutational analysis revealed that this interaction was not mediated by the death domain as the ΔDD construct interacted similarly with full length NRADD. However, deletion of the extracellular domain resulted in a protein that interacted strongly with both forms of NRADD. Similarly, a further deletion construct coding for the intracellular domain alone interacted with full length NRADD. Therefore, the homotypic interaction domain of NRADD is located between the membrane and the DD domain on the intracellular portion.

Fig. 7 A




***NRADD does not interact with other death receptors.*** *Myc tagged NRADD was co-transfected with the indicated FLAG tagged death receptor constructs in 293 cells. Twenty-four hours after transfection in 10 cm dishes, cells were washed twice in PBS and lysed in 1 ml of lysis buffer (20 mM Tris pH 8.0, 137mM NaCl, 10% Glycerol, 1 % NP-40, 5 mM $Na_3VO_4$, 0.5 mM PMSF, and protease inhibitor tablets (Roche)). Lysates were cleared by 10,000 x g spin and incubated with BSA blocked protein G-beads. FLAG beads incubations were done for 3-4 hours at 4°C. Immune complexes were harvested with BSA blocked protein G, washed three times with lysis buffer, then boiled in sample buffer. The eluted proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with αmyc antibodies. Bound antibodies were detected by HRP-conjugated secondary antibodies and ECL detection kit (Amersham). The membrane was stripped and reprobed with αFLAG. * indicates the heavy chain band.*

**Fig. 7B**




***NRADD forms oligomeres.*** *293 cell transfections and co-immunoprecipitations were performed as in Fig. 7A. Interaction with the intracellular domain of NRADD (NRADD ICD-GFP) was assayed by αGFP antibodies.*

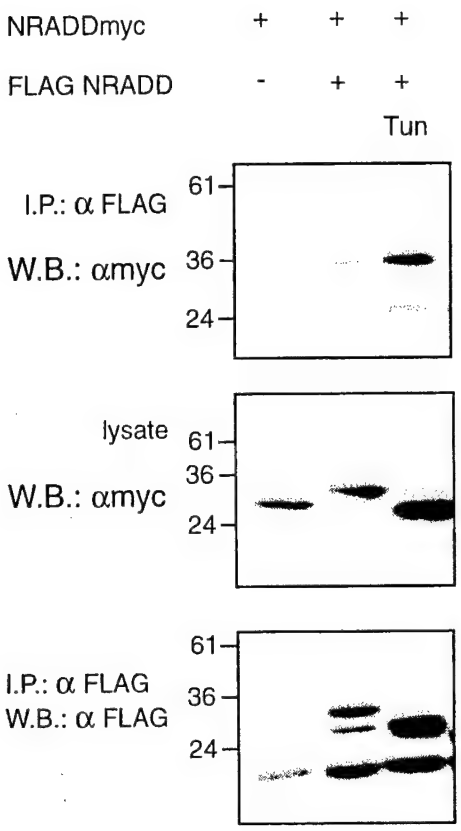
The predicated molecular weight of NRADD is 25 kD. The two NRADD isoforms expressed in 293 cells migrated at ~30 kD and 35kD, respectively. This large shift in mobility is compatible with glycosylation, a post-translational modification frequently observed on death receptors. NRADD contains one consensus N-

glycosylation site (N-X-S/T) in its extracellular domain that is conserved in all 5 mammalian species for which sequences are available. To directly demonstrate that at least part of this shift is due to glycosylation, NRADD was expressed in the presence of tunicamycin, an inhibitor of N-glycosylation. Furthermore, to assess whether the inhibtion of N-glycosylation affected the homo-oligomerziation of NRADD a co-immunuprecipitation experiment was performed (Fig. 8). In the absence of tunicamycin NRADD-myc only weakly associated with FLAG-NRADD. However, upon addition of tunicamycin a drastic increase in the co-IP signal was observed. Tunicamycin also completely abolished the expression of the slower migrating forms of NRADD. Both FLAG-NRADD and NRADD-myc migrate after expression in the presence of tunicamycin as a 30 kD single band indicating the slower migrating isoform is in fact N-glycosylated. The molecular weight of the ΔECD construct is not affected by tunicamycin confirming that N-glycosylation occurs in the extracellular domain (not shown). In this experiment NRADD-myc was mainly expressed as the faster migrating band even in the absence of tunicamycin. This reflects the variability of ratio of the two isoforms observed between different experiments (compare with Fig. 7B).

These interaction experiments indicate that the 48 amino acids in the extracellular domain dominantly inhibit the oligomerization of NRADD. In addition, glycosylated ECDs are required to obtain this inhibition. Dimerization still occurs if only one NRADD molecule contains a glycosylated ECD. The ECD may therefore be critical for the function of NRADD as well.

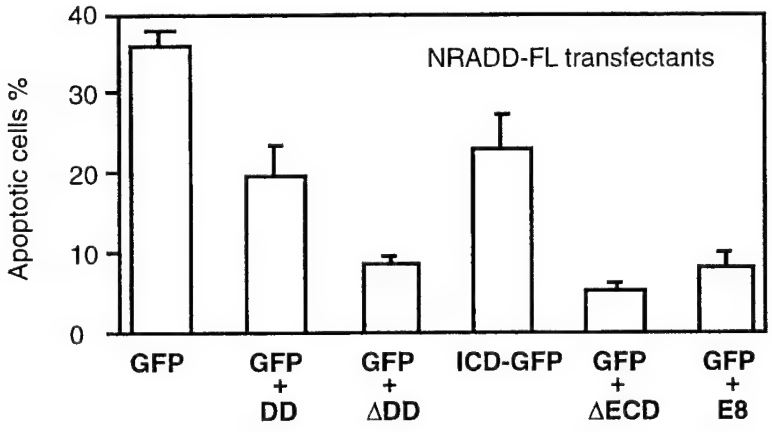
Apoptosis assays in the neuroblastoma line SHEP were performed to test for functional consequences of deleting the ECD. We had previously shown that full length NRADD killed about 25% of these cells in a 24 hr transient expression assay. Expression of the ΔECD construct did not increase cell death above vector control significantly (5%, not shown). Because the ΔECD could interact with full length NRADD apoptotis assays were performed to measure if the ΔECD behaved as a dominant negative. For these experiments conditionally immortalized neurons (ST14A) were used. We had previously shown that expression of wt NRADD kills these cells. Stable transfectants expressing NRADD under the control of a ponasterone inducible promoter were generated. The effect of expressing deletion mutants or inhibitors was assayed in transient experiments using GFP as a transfection marker (Fig. 9). 24 hr ponasterone treatment of GFP transfected cells induced ~40% cells with an apoptotic propidium iodide staining pattern. Co-transfection of the different NRADD deletion mutants caused a decrease in the ponasterone induced killing. Particularly the ΔECD construct protected the cells even more effectively than the vFLIP E8. E8 had previously been found to be one of the strongest inhibitors of the NRADD apoptotic response.

## Fig. 8




***N-glycosylation of NRADD inhibits its oligomerization.*** *Differentially tagged full length NRADD forms were expressed and immunoprecipitated as in Fig. 7. Where indicated tunicamycin (5μg/ml) was added 18 hr prior to lysis.*

**Fig. 9**



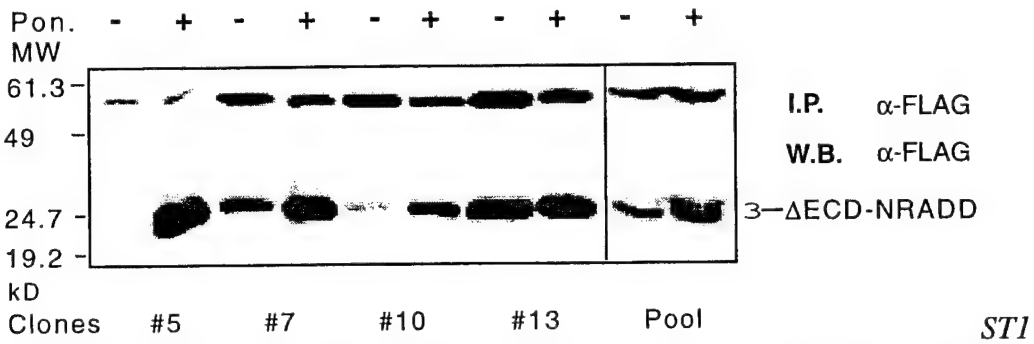

***Protection from NRADD cytotoxicity by some of its deletion mutants.*** *Stably transfected ST14A cells expressing full length NRADD under the control of a ponasterone inducible promoter were transiently transfected with GFP and the indicated constructs. Expression of full length NRADD was induced by ponasterone. 24 ht later cells were fixed and stained with propidium iodide. The DNA staining pattern of the transfected cells (green) were scored under the fluorescence microscope.*

Given the dominant negative effect of the ΔECD construct against full length NRADD stable ST14A cell lines were generated to test if inhibition could also be observed against apoptotic stimuli with characterized physiological targets. ΔECD NRADD-FLAG expressing ST14A cells were generated as above for full length NRADD. The clones and pool generated were analyzed by western blot (Fig. 10 A). The pool revealed some expression in the absence of ponasterone. Analysis of individual clones revealed lines with excellent inducibility and little promoter leakage (#5) while others expressed the deletion mutant constitutively (#13). The survival of constitutively expressing clones is independent conformation that deletion of the ECD abrogates NRADD's pro-apoptotic activity.

The ΔECD expressing cells were challenged with several apoptotic stimuli (Fig. 10B). Stressors for the endoplasmatic reticulum included thapsigargin, tunicamycin, and brefeldin. Cell death was also induced by TNFα in the presence of CHX and exposure to the DNA damaging agent etoposide and UV radiation. Ponasterone addition to these cells was moderately cytotoxic. However in combination with the ER stressors significant protection was observed. In contrast, expression of ΔECD NRADD exasperated the cytotoxicity of TNF/CHX, etoposide, and UV. The interference of ΔECD NRADD with ER mediated apoptotic pathways strongly imply that NRADD is involved in this pathway. The most upstream apoptotic signaling molecules in the ER are currently not known. The full signal transduction mechanism that leads to ER mediated

Caspase-12 activation is also not fully understood. Further studies are needed to define the interaction of NRADD with the other components of this pathway.

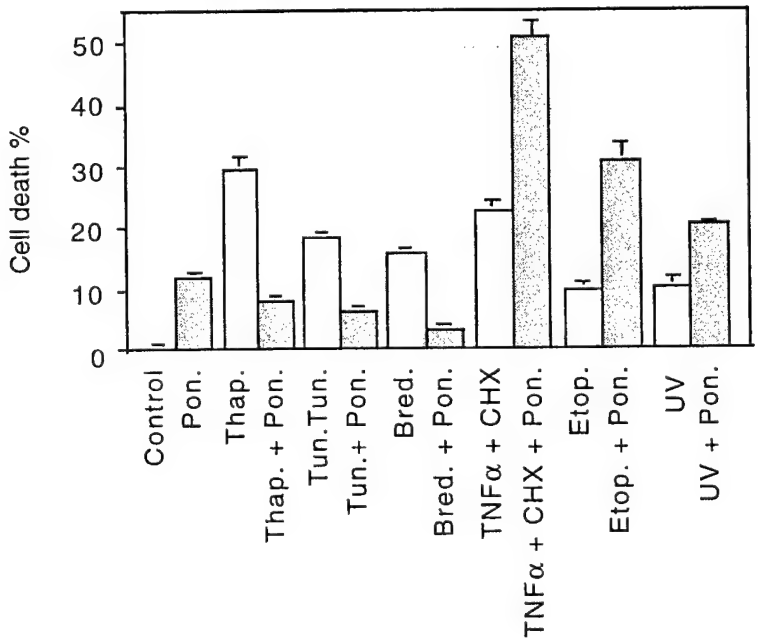
**Fig. 10 A**




*ST1*

*4A cells were transfected with a ΔECD-NRADD FLAG construct in pIND/Hygro and pVgRXR (Invitrogen). Stable transfectants were selected in Hygormycin and Zeocin. For expression analysis ponasterone (15 μm) was added for 48 hr where indicated. Cellular lysates were prepared and analyzed by FLAG immunoprecipitation and western blotting.*

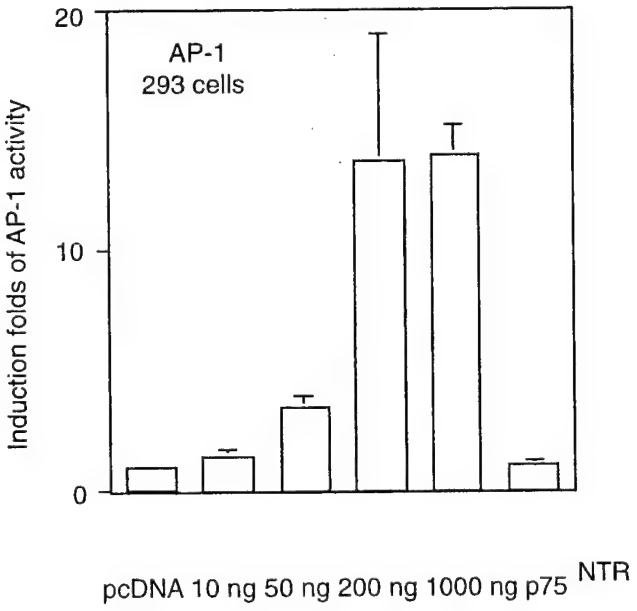
**Fig. 10 B**




*Cells were seeded in 96 wells and treated with the indicated reagents in the presence or absence of 1μM ponasterone for 24 hr. Thapsigargin: 0.3 μM; tunicamycin: 5 μg/ml; TNFα/CHX: 100 ng/ml/10 μM; Etoposide: 0.1 mM; UV: 15 Joules. Cell survival was measured was determined by MTS assay (Pharmacia) according to the instructions of the manufacturer. Cell death was calculated by comparison with non-treated cells.*

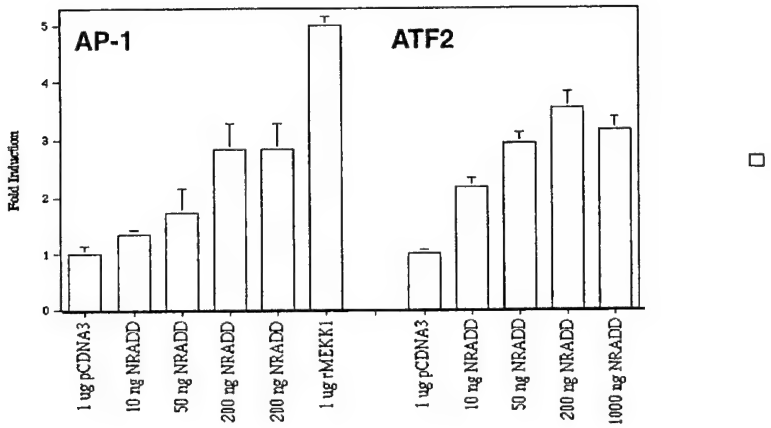
To explore whether NRADD activates other signaling pathways in addition to cell death the Jun pathway was investigated. Co-transfection of an AP-1 luciferase reporter construct with NRADD was used to measure the activation of the pathway (Fig. 11). In 293 cells a 15 fold activation was observed in a dose dependent manner. Interestingly, transfection of $p75^{NTR}$ did not result in significant AP-1 activation in this system indicating that NRADD and $p75^{NTR}$ despite having a very homologous intracellular domain activate different signaling pathways. Analogous experiments were performed in ST14A cells to assess whether NRADD also activates the Jun pathway in cells that are susceptible to NRADD killing. We had previously shown that NRADD does not kill 293 cells. A similar dose dependent AP-1 activation was observed. An ATF2 luciferase reporter was used to assess whether the related p38 pathway was also activated. Again a dose dependent activation was observed. While these studies are not exhaustive they still indicate that NRADD is capable of activating multiple pathways. TNFR1 is a well established example of a death domain containing membrane protein that can activate multiple signaling pathways leading to pleiotropic effects. It is expected that NRADD may also affect different functions depending on the cellular context.

**Fig. 11 A**




***NRADD activates the Jun pathway.*** *293 cells seeded in 6 well plates were transfected by $CaPO_4$ with AP-1 luciferase, pRLTk renilla and the indicated constructs. 24 hrs after transfection the cell lysates were assayed for luciferase and renilla activity using a dual luciferase kit (Promega). Transfection efficiencies were normalized using the renilla values.*

**Fig. 11 B**




*ST14A were transfected with lipofectamine with the reporter constructs AP-1 or ATF2 luciferase together with pRLTk renilla. Included in the transfection mixture were indicated amounts of NRADD or the positive control MEKK1. Luciferase and renilla activities were determined 24 hr after transfection.*

**5) A mouse model to study death receptor function in the central nervous system.**

We had previously reported the generation of transgenic mice expressing the vFLIP, E8, under the control of the prion promoter. E8 inhibits the recruitment of the initiator Caspase-8 to death receptors and consequently inhibits the apoptotic signal from all death receptors. This makes it a convenient tool to distinguish between death receptor mediated apoptosis and apoptosis induced by other pathways. The characterization of the phenotype of these mice would permit a first assessment of the role of death receptors in the CNS during development and for disease states.

We have obtained one line that expresses E8 in the brain, heart, and kidney. These mice show no developmental defects and the microanatomy of the brain is indistinguishable from their wild type littermates. The only phenotype is the development of dermatitis at 3 months of age in starting in the neck region. In addition the spleen is 10 times the weight of the wt littermates. These phenotypes most likely are not related to the transgene expression in the CNS and are therefore not the focus of our study.

In order to identify a phenotype related to CNS mice were challenged with systemic injection of kainic acid. This treatment results in seizures mimicking the excitatory damage induced by glutamine receptors. At a dose of 38 mg/kg kainic acid these seizures last about 4 hr and mortality in a C57B/6 strain is about 80%. When wt and transgenic littermates were compared in their susceptibility to kainic acid a significant protection from mortality was observed ($p=0.03$, $n=4$). Not only did the transgenic mice not die but they also had fewer and less severe seizures. We are currently investigating whether this protection is direct or due to an effect on

inflammatory response. This question is highly significant in the context of multiple sclerosis and the corresponding EAE animal model. In the model the glial and neuronal damage observed are similar to what is observed after and excitatory insult. It is however not understood if the excitatory insult directly causes the cytotoxicity or if it is indirect by activating an inflammatory response. If our experiments show that E8 transgenic brains are protected only from inflammation and not activation of glutamate receptors they would become a valuable tool to identify the target for pharmacological intervention.

**6) Computer modeling of FAS FADD interactions.**

The crystal structure of death domain superfamily heterodimeres where analyzed in detail. The two heterodimer structures solved are teh CARD of Capsase-9 complexed with CARD of Apaf-1 and the death domain of TUBE complexed with the death domain of Pelle. We realized that these very different structures were not mutually exclusive but could be modeled into a single complex. We used this insight to model the FAS-FADD interaction. The result of this theoretical study was published in FEBS letters a reprint of which is attached as an appendix (Weber and Vincenz, 2001).

## Key Research Accomplishments (1996-2001)

- Cloning and characterization of the following components of the death pathway effector machinery.

  Caspase-8 (FLICE)
  Caspase-10 (FLICE2)
  Caspase-13 (ERICE)
  Caspase-14 (MICE)
  RAIDD
  vFLIPs
  cFLIP

- Establish the principle of a caspase cascade.
- Reveal the activation mechanism for upstream caspases.
- Show that wt-Huntingtin is an anti-apoptotic protein acting on the common effector pathway of the apoptotic machinery.
- Develop a cellular system for $p75^{NTR}$ signaling.
- Cloning and characterization of NRADD, a $p75^{NTR}$ homologue.
- Development of transgenic mice with abrogated death receptor function in the central nervous system
- Modeling of a FAS FADD compelx.

## Reportable outcomes (1996-2001)

**Publications supported by this Grant:**

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Duan, H., and Dixit, V. M. (1997). RAIDD is a new 'death' adaptor molecule. Nature *385*, 86-89.

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**Book Chapter:**
Claudius Vincenz. Death receptors and apoptosis: Deadly signaling and evasive tactics. Cardiovascular Disease (2001). **19**, 31-43.

**Meeting Abstracts.**

Keystone meeting on apoptosis: January 2001,Keystone, CO. Abstract #443 & #452.
Characterization of p75NTR apoptotic signaling pathway using a novel cellular model. &
Death domain superfamily interactions: a docking model of a partial DISC complex.

DOD Area of Hope Meeting 2000. Atlanta, GA June 8-11, 2000
Abstract# 51
Inhibition of caspase-9 by Huntingtin

Mechanisms of Cell Death 2000. International Cell Death Society El Escorial, Spain.
May 6-10, 2000
Abstract# 4
Wt-Huntingtin protects from apoptosis upstream of Caspase 3

Keystone meeting Breckenridge, CO. April 6-11, 1999,
Apoptosis and Programmed cell Death.
Abtract# 365
The temporal and spatial Caspase-14, MICE, mRNA expression pattern correlates with areas of programmed cell death in the developing mouse embryo.

AACR Special Conference on the Molecular Mechanisms of Apoptosis Regulation.
Palm Springs, CA. January 9-13, 1998
Abstract# A-63
MICE, a novel short prodomain caspase homologous to Ich-1 (Caspase-2)

**Conclusions**

Only in vitro experiments with recombinant proteins will yield the detailed mechanism by which wtHtt exerts its anti-apoptotic function. We therefore attempted to express the protective domain of Htt in bacteria. These experiments suffered from poor yield and the protein produced did not display the interactions observed with the protein expressed in eukaryotic cells. Therefore, it is likely that the protein is expressed in a non-native conformation. This experimental impasse will have to be overcome by using other expression systems before an in vitro system can be set up to study the inhibition of Caspase-9 activation in vitro.

The complementary in vivo approach was also used in an attempt to confirm the cellular experiments. Studies in the model organism, C. elegans, revealed that wtHtt could be expressed in this organism. Analysis of the transgenic worms bred into several genetic backgrounds revealed no difference in phenotype. This suggests that wtHtt targets a non-conserved step of apical Caspase activation. The activation of the mammalian apical Caspase-9 is significantly different from activation of the C. elegans Caspase-3. The inability of wtHtt to function in C. elegans therefore indicates that the Htt target is the assembly of the apoptosome.

The development of a cellular model to study the apoptotic signaling from $p75^{NTR}$ was brought to a publishable state. This sets the stage to use the model to identify the signaling components that are different between $p75^{NTR}$ and the other death receptors.

Our investigations into the newly discovered $p75^{NTR}$ homologue, NRADD, revealed that oligomerization of this pro-apoptotic is controlled by N-glycosylation. A dominant negative version of NRADD was created that inhibited cell death induced by ER stressors implicating this molecule in apoptotic pathways emanating from the ER.

Towards the understanding of the physiological role of the whole family of death receptors further characterization of E8 transgenic mice revealed that they are protected from excitatory damage. This suggests that while death receptors don't seem to have a role during development they participate in mediating the toxicity leading to neurodegeneration. This mouse model will be an invaluable tool to gauge which neurodegenerative insults may benefit from pharmacological modulation of death receptor activity.

We proposed a new model on how the death domains of FAS and FADD interact in the DISC complex. This theoretical work will provide guidance towards understanding the conformational changes occurring during activation of a death receptor complex.

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## Appendices

**Manuscript:**

Wang, X., Bauer, J.H., Li, Y., Shao, Z., Zetoune, F.S., Cattaneo, E., Vincenz, C. (2001) Characterization of a $p75^{NTR}$ apoptotic signaling pathway using a novel cellular model. J Biol Chem **submitted**.

**Reprints:**

Weber, C. H., and Vincenz, C. (2001). A docking model of key components of the DISC complex: death domain superfamily interactions redefined. FEBS Lett *492*, 171-176.

Claudius Vincenz. Death receptors and apoptosis: Deadly signaling and evasive tactics. Cardiovascular Disease (2001). **19**, 31-43.

# Characterization of a p75$^{NTR}$ Apoptotic Signaling Pathway Using a Novel Cellular Model

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## Summary

The p75 neurotrophin receptor (p75$^{NTR}$) belongs to the tumor necrosis factor TNFR/NGFR receptor superfamily. In some cells derived from neuronal tissues it causes cell death through a poorly characterized pathway. We developed a neuronal system using conditionally immortalized striatal neurons, in which the expression of p75$^{NTR}$ is inducibly controlled by the ecdysone receptor. In these cells p75$^{NTR}$ induces apoptosis through its death domain (DD) in a neurotrophin growth factor (NGF) independent manner. Caspases 9, 6, 3 are activated by receptor expression indicating the activation of the common effector pathway of apoptosis. Cell death is blocked by a dominant negative form of Caspase 9 and Bcl-$X_L$ consistent with a pathway that involves mitochondria. Significantly, the vFLIP E8 protects from p75$^{NTR}$ induced cell death indicating that death effector domains are involved. A p75$^{NTR}$ construct with a deleted DD dominantly interferes with p75$^{NTR}$ signaling, implying that receptor multimerization is required. However, in contrast to the other receptors of the family, p75$^{NTR}$ mediated apoptosis does not involve the adaptor proteins FADD or TRADD, and the apical Caspase 8 is not activated. We conclude that p75$^{NTR}$ signals apoptosis by similar mechanisms as other death receptors but uses different adaptors and apical caspases.

## Introduction

Apoptosis is widespread during development and disease states of the mammalian nervous system (1, 2). The high affinity neurotrophin receptors (Trk) as well as p75$^{NTR}$ have been implicated in this process. Previous studies have shown that the low-affinity NGF receptor p75$^{NTR}$ as well as the high affinity NGF receptor TrkA are widely expressed throughout the central and peripheral nervous system of mammals (3). A few cell types only express p75$^{NTR}$ but not TrkA, such as Schwann cells and oligodendrocytes (4, 5). *In vitro* culture of these cells and exposure to NGF leads to p75$^{NTR}$ induced apoptosis. Furthermore, the pro-apoptotic activity of p75$^{NTR}$ has been documented *in vivo*. Retinal neurons and sympathetic neurons are killed by p75$^{NTR}$ (6, 7). Transgenic expression of the intracellular domain of p75$^{NTR}$ induced apoptosis in selected populations of central and peripheral neurons (8). On the other hand, p75$^{NTR}$ has also been reported to elicit the opposite activity and increase survival leading to the generation, differentiation and maintenance of distinct populations of neurons in mammalian nervous system through modification of TrkA signaling (9).

The neurotrophin family includes NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 and NT-6. p75$^{NTR}$ binds all neurotrophins with low affinity, but ligand induced signaling has only been shown repeatedly for NGF (10) and in single reports for BDNF (7) and NT4 (11).

NGF binding to p75$^{NTR}$ has been reported to trigger NF-κB, activate JNK, and release ceramide (12-14). p75$^{NTR}$ induced apoptosis is NGF dependent in some systems but not others (15, 6). In immortalized cell lines a p75$^{NTR}$ mediated apoptotic response has been reported only in combination with non specific stresses like serum withdrawal, tamoxifen or co-expression of molecules potentially involved p75$^{NTR}$ signaling (15-17).

As a death receptor (DR), p75$^{NTR}$ belongs to the NGF/TNF receptor superfamily. To date, seven DRs have been identified that contain a conserved death domain (DD) (18). This domain has been shown to mediate apoptotic signaling by mediating homotypic interactions

with adaptor proteins (19). While the p75$^{NTR}$ DD displays all the structural hallmarks of a DD no homotypic interaction partners have been identified so far (20).

In contrast, the Fas and TNFR1-death pathways are well understood and involve receptor activation by their cognate ligands, FasL and TNF-$\alpha$, respectively. Upon activation, the adaptor-proteins FADD and FADD/TRADD are recruited and activate Caspase 8. Activated Caspase 8 initiates proteolytic activities of other downstream caspases like Caspase 3 and Caspase 6, which then cleave specific substrates resulting in apoptotic morphology and DNA fragmentation (19).

The apoptotic signaling mechanism of p75$^{NTR}$ is not understood in such detail. However, caspase inhibitors and Bcl-$X_L$ inhibit the process suggesting that the downstream components are conserved (21, 22). The proteins SC-1, NRIF, NADE, ERKs, FAP-1, Caveolin-1, NRAGE, as well as the GTPase RhoA have all been reported to bind the intracellular domain of p75$^{NTR}$ and will potentially provide some insight into the signaling cascades triggered by this receptor (23-28, 16, 29). However, it is currently not understood how any of these molecules are able to transmit the signal to the apoptotic effector machinery.

Cellular systems have been instrumental to elucidate signaling from other death receptors such as FAS and TNFR1. To undertake similar studies we developed a cellular system as a tool to study p75$^{NTR}$ induced apoptotic signaling. p75$^{NTR}$ receptor expression in ST14A cells was sufficient to induce cell death without the confounding effect of co-stimuli. ST14A cells are conditionally immortalized neurons derived from the striatum and retain the expression of multiple markers characteristic for neurons but in addition are highly transfectable and immortalized at the permissive temperature (30).

Using this system we show that the DD is essential for the initiation of apoptosis. Truncation mutants without DD are inactive and dominantly interfere with p75$^{NTR}$ signaling. The similarities to other DRs also included the inhibition by the viral protein E8, activation of Caspase 9, and multiple downstream caspases. However, Caspase 8 was not activated and FADD DN, did not have any inhibitory effect. Hence, p75$^{NTR}$ induces apoptosis in ST14A cells

through a similar molecular mechanism to that of other DRs but via different adaptors and apical caspases.

## Experimental Procedures

*Reagents and antibodies*-Zeocin and ponasterone were obtained from Invitrogen (Carlsbad, CA), hygromycin B from Roche (Indianapolis, IN), lipofectamine from GIBCO BRL (Gathersburg, MD), NGF from Harlan Bioproducts (Madison, WI). Caspases substrates were from Stratagene (La Jolla, CA). The BrdU kit was from BD PharMingen (San Diego, CA). Propidium iodide (PI), poly-L-lysine hydrobromide, protein G-sepharose, α-FLAG M2 antibody and anti-mouse IgG FITC conjugate (Fc specific) were from Sigma (St. Louis, MO). α-NGFR polyclonal antibody was from Promega (Madison, WI). Secondary antibodies and ECL reagents were from Amersham Life Science (Piscataway, NJ).

*Cell Lines, expression vectors and transfections*-ST14A are striatal neurons conditionally immortalized by transfection with a temperature sensitive form of the SV-40 large T antigen (30). ST14A and 293 human embryonic kidney cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% L-glutamine, and penicillin/streptomycin in 5% $CO_2$ at 33 °C (for ST14A cells) or 37 °C (for 293 cells). Human neuroblastoma cells, SK-N-BE, were grown at 37 °C in MEM/Hams F-12 nutrient mixture (1:1) with the same additions as for ST14A cells. Schwann cells were isolated from sciatic nerve of neonatal rats, single cell suspension was achieved by trypsin and collagenase, and cultured with 10% fetal bovine serum, 6 mM L-glutamine, penicillin/streptomycin, 20 μg/ml pituitary extract and 2 μM forskolin on poly-L-lysine–coated tissue culture dishes at 37 °C. A-431 carcinoma cells were grown in DMEM/F 12 (1:1) medium containing 2.0 mM glutamine, 100 units/ml of penicillin, 0.1 mg/ml streptomycin, 0.25 mg/ml amphotericin B and 5% fetal bovine serum at 37 °C.

The p75$^{NTR}$ constructs shown in Fig. 1 were obtained by PCR and cloned into the ecdysone-inducible mammalian expression vector pIND-Hygro (Invitrogen) using a human p75$^{NTR}$ plasmid kindly provided by Moses V. Chao (New York University). All constructs were verified by sequencing and ponasterone inducible expression in 293 cells. For stable

expression, p75$^{NTR}$ constructs were transfected together with the ecdysone receptor plasmid pVgRXR. Cells expressing p75 (ST14A-p75$^{ind}$) were selected by growth in hygromycin B (0.625 mg/ml) and zeocin (0.625 mg/ml) and screened for expression by immunoblotting.

*RT-PCR*-Total RNA (1 μg) was isolated from the different cell lines using Trizol reagent (GIBCO BRL). After reverse-transcription, cDNA was amplified using superscript one-step RT-PCR system (GIBCO BRL). For RT-PCR, the following primers were used: rat p75$^{NTR}$, 5'-AGCCAACCAGACCGTGTGTG-3' and 5'-TTGCAGCTGTTCCACCTCTT-3' (31); rat TrkA, 5'-GCTGACCAATGAGACCATGCGGCAT-3' and 5'-GTGAGCAGCTCTG CCTCACGATGG-3' (31); rat GAPDH, 5'-ATGGTGAAGGTCGGTGTCAACGGA-3' and 5'-TTACTCCTTGGAGGCCATGTAGGC-3'.

*MTS assay*-The assay was performed according to the manufacturer's instructions (Promega, Madison, WI). About 5 x10$^4$ cells grown in a 96 well-plate in 100 μl medium were either left untreated or treated with ponasterone. Twenty μl of combined MTS/PBS solution was added and the cells were put in the incubator for 1 hr before reading the absorbance at 490 nm.

*DNA fragmentation assay*-About 5 x 10$^6$ ST14A cells treated with ponasterone for different times were harvested by DNA Zol (MRC, Ohio). DNA was purified by EtOH precipitation. The precipitate was dissolved in $H_2O$, digested 30 min at 37 °C with 20 μg/ml DNAse-free RNAse and analyzed by electrophoresis on a 1% agarose gel.

*Measuring p75$^{NTR}$ expression by FACS analysis*-About 5 x 10$^6$ ST14A cells were treated with ponasterone or left untreated. Attached cells were harvested in 135 mM potassium chloride/15 mM sodium citrate at 37°C for 5 min. The resulting cell suspensions were pooled with cells in the supernatant, washed with 1% heat-inactivated fetal bovine serum in PBS, blocked with 3% BSA in PBS for 30 min, and incubated with α-FLAG-, or α-p75$^{NTR}$ -

antibody (1: 100) for 1.5 h and FITC-conjugated secondary antibody (1: 2000) for 1 h. All processing was performed at 4°C. The cells were analyzed on a Beckman flow cytometer.

*Immunoprecipitation and immunoblotting*-About 5 x $10^6$ cells were collected in lysis buffer (20 mM Tris pH 8.0, 137 mM NaCL, 10% glycerol, 1% NP40, 2 mM EDTA with 5 mM Na2VO4, protease inhibitor cocktail (Roche), 0.2 mM PMSF) on ice and directly analyzed by Western blot or immunoprecipitated after clearing at 10,000 x g for 10 min. The soluble lysates were incubated with the relevant antibodies for 3 h and immune complexes were harvested with protein G-sepharose beads for 1 h, followed by three washes in lysis buffer, and boiling in sample buffer. Eluted proteins were separated on SDS-PAGE, and analyzed by Western blot (1:1000 dilution of primary and 1:10000 of secondary antibodies).

*Microscopic determination of cell death*-About 1 x $10^6$ ST14A-p75$^{ind}$ cells were grown on glass coverslips coated with gelatin and co-transfected with plasmids expressing the indicated proteins (1 μg) and GFP as a transfection marker (0.25 μg). After transfection, cells were treated with ponasterone for 48 h, washed with PBS and fixed with 4% paraformaldehyde, incubated 10 min in 0.5 μg/ml PI, and washed with PBS. Transfected (green) cells were scored by fluorescence microscopy for chromatin condensation and nuclear fragmentation revealed by intense PI staining.

*TUNEL assay*-About 1 x $10^6$ ST14A-p75$^{ind}$ cells were transfected as above and treated with ponasterone for 48 h. Attached cells and matching supernatant were pooled, fixed with 4% paraformaldehyde, and submitted to TUNEL (terminal deoxy-transferase-mediated Br-dUTP nick end labeling) using PE-conjugated anti-BrdU monoclonal antibody flow cytometer following the instructions of the manufacturer. Labeled cells were analyzed on a Beckman flow cytometer.

*Caspase activity assays*-Cell extracts and enzyme assays were performed as previously described (32). Briefly, 5 x $10^6$ cells were harvested in PBS, and homogenized with a Dounce homogenizer in extraction buffer (10 mM KCl, 1 mM DTT, 5 mM EGTA, 25% glycerol, 1

µg/ml each of Leupeptin, aprotinin, soybean trypsin inhibitor and pepstatin, 0.2 mM PMSF in 10 mM Hepes pH 7.4) with 40 strokes. Twenty µg of lysate proteins in 100 µl final volume were incubated with 0.2 mM of the following Caspase substrates at 37 °C for 3 h. Caspase 1: Ac-YVAD-AFC; Caspase 3: Ac-DEVD-AFC; Caspase 6: Z-VEID-AFC; Caspase 8: Ac-IETD-AFC; Caspase 9: Ac-LEHD-MCA. Caspases substrates incubated in non-ponasterone treated extracts were used as control. Released AFC or MCA was read in a cytofluor II fluorescence multi-well plate reader (excitation at 400 nm and emission at 505 nm for AFC labeled substrates, or excitation at 360 nm and emission at 460 nm for the MCA substrate).

*NGF measurements*- The ELISA kit from Promega (Madison, WI) was used to determine NGF in the media according to the instructions of the manufacturer. Cells were incubated in serum free media supplemented with 1% BSA for 24 h and 48 h prior to the assay.

*Statistical analysis*- StatView software was used to calculate significant differences by ANOVA algorithm. Error bars indicate standard errors (S.E.).

## Results

*p75 induces cell death in ST14A cells*-Multiple functional studies on p75$^{NTR}$ have been performed using primary neuronal cells and transgenic or knockout models (33). These studies show that p75$^{NTR}$ engages multiple and sometimes opposing signaling pathways, depending on the cell type, microenvironment, and stage of development (10). Invariably, cellular systems developed to study p75$^{NTR}$ function and mechanisms show only a subset of the responses attributed to p75$^{NTR}$ signaling.

In search of a model for the apoptotic signaling of p75$^{NTR}$ we investigated the ST14A cell line. These cells are derived from the striatum of embryonic day 14 rats and are conditionally immortalized by expression of the temperature sensitive mutant of the SV40 Large T Antigen (30). ST14A cells acquire antigenic and electrophysiological properties characteristic of mature neurons and become postmitotic at the non-permissive temperature (39°C) (34). Because these neuronal cells are susceptible to a variety of apoptotic stimuli, including the death receptor TNFR1 (35), we investigated their responses to p75$^{NTR}$.

Stable ST14A expressing p75$^{NTR}$ lines (ST14A-p75$^{ind}$) were established using an ecdysone inducible system to avert problems with the pro-apoptotic activity of p75$^{NTR}$ during the selection process. This two-plasmid system puts the gene of interest under the control of the Ecdysone/GRE promoter. The ecdysone receptor is transcribed from the co-transfected pVgRXR plasmid and expression of the gene of interest is induced by addition of the ecdysone homologue ponasterone. Expression of the two NGF receptors in parental ST14A as well as lines carrying an expression vector for p75$^{NTR}$ was assayed by RT-PCR (Fig. 2A). Parental ST14A cells were negative for p75$^{NTR}$ but expressed similar levels of TrkA as the carcinoma cell line A431. Exposing these cells to ponasterone had no effect on the levels of NGF receptors (lanes 3,4). Analysis of the ST14A-p75$^{ind}$ cells revealed expression of p75$^{NTR}$ RNA both in a pool and two randomly selected clones (lanes 6-11). A robust induction of p75$^{NTR}$ by ponasterone was observed after 15 PCR cycles (lanes 5,6). Amplification of the RT-product for 30 cycles yielded also a band in the absence of ponasterone indicating some leakiness of the

promoter (lanes 7,9). The levels of expression were lower than endogenous p75$^{NTR}$ levels in Schwann cells (lane 11). TrkA levels remained unchanged in the transfectants with or without ponasterone. These results indicate that the ST14A clone used here did not express endogenous p75$^{NTR}$ and therefore represents a convenient null background for the introduction of wild type or mutants.

Addition of ponasterone to ST14A-p75$^{ind}$ cells produced dramatic cell killing as demonstrated by measuring viability with the MTS assay (Fig. 2B). After a 2 day treatment with ponasterone at 33 °C about 75% of mitochondrial activity was lost in freshly established ST14A-p75$^{ind}$ compared to the untreated cells. The cells detach from the substrate, round up and develop cell membrane blebs during the treatment (not shown). However, ST14A-p75$^{ind}$ that were kept in culture for extended periods of time gradually became resistant to ponasterone at 33 °C (passage # 40). Ponasterone was not cytotoxic when added to parental ST14A cells.

To demonstrate expression of the exogenous protein on the cell surface FACS analysis was performed on p75$^{NTR}$-FLAG transfectants (Fig. 2C). The epitope tag in this construct is engineered immediately C-terminal to the signal peptide (Fig. 1). Non-permeabilized cells were stained with α-FLAG antibodies with or without ponasterone treatment. Fluorescence in the absence of ponasterone was identical to the background control. After overnight incubation with ponasterone increased staining of cells with α-FLAG was measured. In contrast, late passage cells did not show any expression at 33 °C. However, at 39 °C expression as well as sensitization to cell killing by ponasterone was restored (not shown). To avoid these confounding factors of prolonged culture all subsequent experiments were performed on cells passaged less than 20 times unless indicated.

Because in some systems NGF is able to induce cell death by binding to p75$^{NTR}$ we investigated whether addition of this neurotrophin affects the cell death response in ST14A-p75$^{ind}$. The loss of viability of ST14A-p75$^{ind}$ upon ponasterone treatment was not affected by incubation in serum free media indicating that the process was not controlled by serum derived

factors (Fig. 3A). Similarly, cell killing induced by ponasterone was neither enhanced nor inhibited by the addition of NGF (10 ng/ml) as measured by MTS assay (Fig. 3A). To investigate a possible autocrine mechanism NGF levels in the media of parental and ST14A-p75$^{ind}$ were measured by ELISA. After 48 h incubation of parental ST14A cells 75.25 ± 19 pg/ml and 39.38 ± 17.41 pg/ml NGF was detected in the absence and presence of ponasterone, respectively. The p75$^{NTR}$ transfectants produced similarly low levels of NGF (29.5 ± 4.91pg/ml). These levels of NGF are in the picomolar range well below the nanomolar concentrations required for p75$^{NTR}$ activation. Therefore, a NGF autocrine mechanism cannot account for the p75$^{NTR}$ induced cell death in ST14A-p75$^{ind}$.

NF-κB activation in ST14A-p75$^{ind}$ cells was also measured. No significant induction of this pathway could be observed using luciferase reporter assays (not shown), suggesting that p75$^{NTR}$ cannot activate NF-κB in these cells or that the strong pro-apoptotic response masks other signals that potentially emanate from this receptor.

The intracellular domain of p75$^{NTR}$ contains a well conserved DD whose role in initiating apoptosis has been disputed (21, 36). To address this issue in the ST14A system we established ST14A-p75$^{ind}$ lines expressing FLAG tagged full length and deletion mutants (Fig. 1). Deletions of the DD (ΔDD) and the whole intracellular domain (ΔICD) were tested. Expression of both the ΔDD and ΔICD had no effect on cell viability similar to parental ST14A cells (Fig. 3A). Levels of expression were measured by immunoblotting with α FLAG antibodies (Fig. 3B). Ponasterone induced the expression of a doublet band at ~75 kD in the FL transfectants. This is the molecular weight reported for the glycosylated form of endogenous p75$^{NTR}$ (37). Immunoprecipitation with p75$^{NTR}$ specific antibodies revealed that the levels of p75$^{NTR}$ expressed were slightly lower than endogenous p75$^{NTR}$ in Schwann cells. A similar level of expression was seen when lysates from cells expressing the ΔDD and ΔICD constructs were probed together with the full length p75$^{NTR}$ lysates. Doublet bands at ~65 kD

and 45 kD were induced by ponasterone with the ΔDD and ΔICD, respectively. The transfectants expressing the truncation mutants were also analyzed by FACS for cell surface expression using the α-p75$^{NTR}$ antibody. More than 75% of the cells stained positive after overnight incubation with ponasterone (not shown). Therefore, the failure of the ΔDD and ΔICD to induce apoptosis is due to the inability of these mutants to signal cell death and not due to lack of expression or inappropriate cellular localization. In these cells, therefore, deletion of the DD impairs the ability of p75$^{NTR}$ to signal cell death. Shown here are the results from transfection pools but the analysis of 7 ΔDD and 3 ΔICD expressing clones produced identical results.

*p75$^{NTR}$ induces apoptosis*-The size distribution of DNA recovered from ST14A cells was analyzed in order to establish whether p75$^{NTR}$ induced cell death proceeded by an apoptotic mechanism (Fig. 6A). Treatment of the ST14A-p75$^{ind}$ cells with ponasterone induced degradation of DNA into a pattern of approximately 200 bp periodicity that was particularly distinct after 48h when most cells have acquired apoptotic morphology. This degradation was not observed without ponasterone treatment. Such an oligonucleosomal pattern is a prominent feature of apoptosis and therefore indicates that p75$^{NTR}$ is able to activate an apoptotic pathway. Consistent with the observation that the ΔDD and ΔICD were not cytotoxic no DNA degradation was observed upon ponasterone induction of those cell lines.

The appearance of an oligonucleosomal ladder indicated that DNAses were activated by p75$^{NTR}$. Because the activity of apoptotic DNAses is regulated by caspases (38), we attempted to identify which Caspases participate in the execution of p75$^{NTR}$ induced apoptosis. Cellular extracts were prepared after treatment with ponasterone and caspase activities were measured using specific fluorogenic tetrapeptide substrates. Robust induction of Caspase 9 activity as well as of the downstream Caspases 3 and 6 was observed. The Caspase 1 substrate was also cleaved efficiently in the ponasterone activated extract (Fig. 4B). Significantly, no Caspase 8

activity was induced. To verify that Caspase 8 can be activated and its activity measured by the assay, a control experiment was performed (inset). The cells were either transfected with TNFR1 or treated with TNF-α in the presence of cycloheximide. The Caspase 8 substrate was cleaved when added to these extracts demonstrating that the assay detects activation of this caspase in this cell line. Therefore, the absence of Caspase 8 activity in ST14A-p75$^{ind}$ cells suggests that Caspase 8 is not involved in p75$^{NTR}$ induced apoptosis, and implies that p75$^{NTR}$ signal transduction activates a different apical Caspase than FAS or TNFR1 in ST14A cells. The control ST14A cell line expressing ΔICD p75$^{NTR}$ did not induce Caspase 9 activity upon ponasterone treatment, consistent with the inability of this truncation mutant to induce apoptosis. Moreover, cells expressing the ΔDD construct did not respond to ponasterone treatment with increased caspase activity either, further documenting the proapoptotic activity of this domain.

*p75$^{NTR}$ induced apoptosis is repressed by general and DR specific inhibitors*-The ST14A system made it feasible to delineate which components of the apoptotic machinery are involved in p75$^{NTR}$ mediated apoptosis. Several anti-apoptotic molecules with specific molecular targets that occur naturally have been identified or have been engineered. The effect of these inhibitors on the p75$^{NTR}$ apoptotic pathway was studied (Fig. 5). ST14A-p75$^{ind}$ cells were co-transfected with a plasmid expressing the indicated inhibitor and GFP as a transfection marker. The PI staining pattern of the transfected cells was evaluated by fluorescence microscopy after 24 h of ponasterone treatment (Fig. 5A). Vector and GFP alone induced a background of an apoptotic staining pattern of ~7%. Treatment with ponasterone increased this value to 22%, which is an underestimate as floating cells are lost to analysis in this assay.

We tested E8 and FADD DN as inhibitors specific for DRs (39-42). Expression of E8, an equine herpesvirus protein consisting of two death effector domains, effectively inhibited the apoptotic response. In contrast, transfection of a FADD DN, a construct containing only a

DD and missing its DED, did not reduce the apoptotic response. This is surprising, as FADD DN, like E8, inhibits signaling from other DRs. TRADD DN, an inhibitor specific for TNFR1 (43) induced apoptosis, also had no effect.

To ascertain that the dominant negative forms of FADD and TRADD were active in ST14A cells a control experiment was performed using transfection of TNFR1 as the pro-apoptotic stimulus (Fig. 5A, inset). Expression of both molecules inhibited TNFR1 induced cell death as effectively as observed in non-neuronal cells.

To establish whether mitochondria were involved in mediating or amplifying the p75$^{NTR}$ signal, we tested Caspase 9 DN and Bcl-$X_L$. Anti-apoptotic Bcl-2 homologues act by inhibiting the release of pro-apoptotic molecules, such as cytochrome c, from mitochondria, while Caspase 9 DN inhibits the ensuing Caspase 9 activation at the apoptosome (44, 45). Caspase 9 DN and Bcl-$X_L$ were both effective inhibitors and reduced the number of cells with a dense PI staining pattern to almost background levels.

CrmA is a viral caspase inhibitor with highest affinity for Caspase 1, followed by Caspase 8 (46). CrmA, too, was able to mediate substantial inhibition of cell death, which is consistent with our finding that a Caspase 1-like activity is induced (Fig. 4).

To obtain independent confirmation of these results we analyzed ST14A-p75$^{ind}$ cells using TUNEL as an alternative method to measure apoptosis. Cells were transfected and p75$^{NTR}$ was induced as above but analysis was performed by labeling DNA ends with BrdU. The amount of apoptosis was determined by measuring the TUNEL and GFP double-positive cells (Fig. 5B). Almost identical results were obtained using this unbiased approach as with the microscopic evaluation.

*p75$^{NTR}$ signaling requires receptor multimerization*-A prerequisite for DR signaling is the oligomerization of the receptor. The prototype DR, TNFR1, exists as a trimer. Similarly Fas is also oligomerized (47, 48). Recently it has been shown that TNFR1 and FAS already pre-exist on the cell surface as oligomers before ligand binding (49, 50). Because of this

multimerization requirement, expression of deleterious receptor mutations leads to a DN phenotype as long as they still multimerize (50). To further investigate if p75$^{NTR}$ has similar requirements for receptor homo-oligomerization as other DRs, we tested the C-terminal truncation mutants for a DN effect (Fig. 8a). The ΔDD and the ΔICD p75$^{NTR}$ constructs (Fig.1) were co-transfected into ST14A-p75$^{ind}$ cells together with GFP marker. Wild type p75$^{NTR}$ was induced by the addition of ponasterone. Microscopic evaluation showed that p75$^{NTR}$ without a DD acted as a strong DN and inhibited apoptosis as effectively as E8. Deletion of the entire intracellular domain did not produce a molecule with protective properties. Western blot analysis revealed that induction of p75$^{NTR}$ was similar in all transfections indicating that the inhibitors affected directly p75$^{NTR}$ signaling and not its expression. Co-immunoprecipitation experiments were performed to assess the binding potential of the deletion constructs to full length p75$^{NTR}$ (Fig. 6b). FLAG tagged p75$^{NTR}$ full length and deletion constructs were co-expressed in 293 cells together with full length p75$^{NTR}$-GFP. FLAG immunoprecipitation and Western blotting with α-GFP revealed that full length, ΔDD, and ΔICD p75$^{NTR}$ were all able to associate with p75$^{NTR}$-FL-GFP. p75$^{NTR}$-GFP did not associate non-specifically with FLAG-beads. Therefore, p75$^{NTR}$, like other receptors of this family, was able to self associate through the extracellular domain and incorporation of signaling defective mutants into the complex leads to its inactivation.

## Discussion

*A new cellular model*-The ST14A model presented here is the only model where a strong apoptotic response is elicited by p75$^{NTR}$ alone without any additional stimuli. ST14A cells are effectively killed by induced expression of p75$^{NTR}$. Interestingly, addition of NGF had no effect. Endogenous levels of NGF secreted into the media are well below the affinity of p75$^{NTR}$ excluding an autocrine mechanism. These observations are in support of the hypothesis that p75$^{NTR}$ signals in the absence of ligand in some cells (15). ST14A-p75$^{ind}$ cells were not rescued by the addition of NGF suggesting that addition of ligand does not negate the pro-apoptotic activity of p75$^{NTR}$. TrkA on ST14A cells is also insufficient to prevent apoptosis, even though TrkA was expressed on ST14A-p75$^{ind}$cells and was activated by NGF as assayed by measuring NGF induced tyrosine autophosphorylation (not shown).

The large T-antigen has been reported to inhibit apoptosis by death receptors, serum starvation and caspase-1 activation, raising the possibility that the immortalization of ST14A will interfere with apoptotic signaling (51-53). The observation, however, that p75$^{NTR}$ induced cell death is unimpeded at the non-permissive temperature of 39 °C implies that the pro-apoptotic responses are independent of the large T-antigen. The development of resistance after extensive passaging of the cells is potentially mediated by the large T-antigen as sensitivity and expression was regained upon shifting to the non-permissive temperature. Further studies are needed to evaluate the role of the large T-antigen for the development of resistance.

Expression of the receptor in ST14A-p75$^{ind}$cells is comparable to endogenous levels of p75$^{NTR}$ in Schwann cells (Fig. 3B) suggesting that our system is relevant to *in vivo* situations. The inducible system permits a regulatable expression of p75$^{NTR}$ and induction of cell death. It is therefore less susceptible to artifacts during the selection of stable lines, which is also confirmed by the uniformity of the ponasterone effect in single clones versus pools. ST14A-p75$^{ind}$ cells therefore represent an example of a neuronal cell type where both NGF receptors are expressed and p75$^{NTR}$ signals cell death in a dominant and ligand independent manner. Similar p75$^{NTR}$ dominant signaling has also been observed *in vivo* in peripheral nerves where

the ICD of p75$^{NTR}$ is capable of inducing cell death, even in the presence of neurotrophic support and TrkA (8).

*The apoptotic signaling mechanism of p75$^{NTR}$*-Deletion mutagenesis shows that the DD of p75$^{NTR}$ is required for the induction of apoptosis (Fig. 3). The ΔDD construct not only is unable to induce apoptosis but also interferes dominantly with apoptosis induced by wt-p75$^{NTR}$ (Fig. 6). This indicates that cell death induction by p75$^{NTR}$ is similar to other DRs, which all use this domain to recruit DD containing adaptor molecules (54). Extensive searches for p75$^{NTR}$ interacting molecules have identified nine proteins that interact with the intracellular domain of p75$^{NTR}$ (23-28, 16, 29, 12). None of these proteins, however, contain a DD and NADE is the only protein implicated in cell death that interacts with the DD of p75$^{NTR}$ (25). The p75$^{NTR}$-DD structure contains all the marks of a typical DD and falls well within the structural variation observed within this family. No features have been identified that would prevent this domain to function as a homotypic interaction domain (55). The fact that the isolated p75$^{NTR}$ DD does not form homodimers in solution has been used for the argument that this DD does not provide an interaction surface. However, other death domains with known binding partners also do not form homo or heterodimers in solution when expressed as isolated domains (56). Therefore, homotypic interactions partners for the p75$^{NTR}$ DD may still be identified.

Experiments performed in sensory neurons have indicated that cell death can be induced by truncated p75$^{NTR}$ without a DD (21). In this system a membrane anchored juxtamembrane domain was sufficient to induce cell death (36). Considering the multitude of effects p75$^{NTR}$ elicits it is possible that sensory neurons signal cell death by a different mechanism than ST14A cells.

The equine herpesvirus E8 is a strong inhibitor of p75$^{NTR}$ induced apoptosis (Fig. 5). E8 acts by precluding the recruitment of Caspase 8 to the death inducing signaling complex (DISC) (39-42). Since we were unable to detect Caspase 8 activation, we hypothesize that a

different caspase is recruited by p75$^{NTR}$ in ST14A cells (Fig. 4B). In support of this hypothesis, Gu et al. reported that Caspase 8 was not processed by NGF triggered apoptosis in oligodendrocytes (22). This is further confirmed by the inability of FADD DN to interfere with p75$^{NTR}$ signaling. Similarly to E8, FADD DN inhibits recruitment of both Caspase 8 and Caspase 10 to the DISC (57). The involvement of different apical Caspases and adaptor proteins is furthermore indicated by the observation that a fusion protein between the extracellular domain of FAS and the intracellular domain of p75$^{NTR}$ is not able to kill cells that readily die by expression of FAS (58). Nonetheless, the E8 results indicate that death effector domain interactions are essential for p75$^{NTR}$ signaling, much as they are needed for signaling from the other DRs. However, the proteins containing these DEDs still need to be identified.

Cleavage of the Caspase 1 substrate was induced by p75$^{NTR}$ expression. The main role of Caspase 1 is to cleave pro IL-1 and not to mediate apoptosis (59). However, Caspase 1 has the same substrate specificity as Caspases 4 and 5 and they are both capable of inducing apoptosis (60, 61). The effective inhibition observed with CrmA confirms the involvement of this group of Caspases.

Cells have been subdivided into type I or type II groups depending on the apoptotic mechanisms induced by FAS (62). Type I cells have high levels of DISC components and apoptosis is not inhibited by Bcl-$X_L$. Type II cells generate a weaker DISC activity that requires the mitochondrial amplification loop to induce apoptosis and Bcl-$X_L$ protects in these cells. Given the inhibition by Bcl-$X_L$ and Caspase-9 DN the p75$^{NTR}$-ST14A cells described here belong to the type II group of cells.

Similar to other DRs, ΔDD-p75$^{NTR}$ is defective in signaling and acts as a DN, implying that multimerization of p75$^{NTR}$ is required for signaling. The ΔICD however is able to multimerize but does not inhibit signaling. Possibly, the extracellular domain is unable to affect the conformation of the intracellular domains of its full length interaction partners or a mutated intracellular fragments is required to interfere with the assembly of a functional

receptor signaling complex. p75$^{NTR}$ has also been shown to functionally and physically interact with the high affinity neurotrophin receptors (reviewed in (10)). These heterotypic interactions are mediated by both the intracellular and the extracellular domains of both receptors. Interestingly deletion of the ICD leads to an enhancement of TrkA basal signalling (63). This suggests that pro-apoptotic signaling through homotypic interactions of p75$^{NTR}$ involves different p75$^{NTR}$ domains than anti-apoptotic signaling through heterotypic interactions with the Trk receptors.

The primary sequence is not conserved well enough in the DR family to be able to identify a pre-ligand-binding assembly domain (PLAD) on p75$^{NTR}$ that has been shown on FAS and TNFR1 (49, 50). However, the ability of the ΔICD mutant to interact with p75$^{NTR}$-FL indicates that the extracellular domain is important for homotypic interactions.

Our results show that p75$^{NTR}$ apoptotic signaling has similarities with other DRs. The ST14A model does not recapitulate all p75$^{NTR}$ signals but its strong apoptotic response provides the means to study DD dependent signaling from this receptor. In particular, detailed structure guided mutational studies will provide further information on the DD interaction interface necessary to generate the apoptotic signal. This information will be valuable for evaluating which of the multiple p75$^{NTR}$ interacting proteins participate in the DD dependent signaling and in the rational design of specific inhibitors. The cellular model presented here is suited to evaluate the pharmacological potential of small molecules or engineered proteins. The specificity of these reagents can also be assessed as the cells are susceptible to killing by other DRs such as TNFR1.

Even though we developed ST14A-p75$^{ind}$ primarily as a tool for mechanistic studies, results obtained with cells are likely to be directly applicable to striatal neurons *in vivo*. The ST14A clone used in this study did not express endogenous p75$^{NTR}$ (Fig. 2) contrary to a previous report, indicating clonal variability (30). Expression of p75$^{NTR}$ in the rat striatum *in vivo* has only been shown in cholinergic neurons (64). However, the majority of the striatal neurons are GABAergic expressing DARPP-32. ST14A cells also express DARPP-32 and are

therefore more similar to the bulk of the striatal neurons (34). Expression of p75$^{NTR}$ in DARPP-32 positive cells is not observed during development. However, ischemia has been shown to induce p75$^{NTR}$ expression in the resistant cholingergic striatal neurons (65). In addition, striatal neurons have been shown to undergo apoptosis after transient focal ischemia (66). Possibly, expression of p75$^{NTR}$ also occurs in the GABAergic neurons early in the course of ischemia but are rapidly killed similarly to the ST14A cells presented here. The development of specific inhibitors for p75$^{NTR}$ is needed to ascertain the therapeutic benefit of modifying the activity of p75$^{NTR}$ for a variety of disease states. Multiple studies have indicated a possible role of this receptor in acute neuronal injuries and progressive neurodegenerative disorders. p75$^{NTR}$ expression is upregulated after axotomy or neuronal injury and p75$^{NTR}$ antisense oligonucleotides have been shown to reduce the damage (67-69). The β-amyloid peptide that is accumulating as extracellular deposits during the progression of Alzheimer's disease has been shown to bind and activate p75$^{NTR}$ (70-71). The cellular model presented here will be a valuable tool for mass screening of anti-apoptotic compounds that may have a benefit to treat these and perhaps other neurological disorders where p75$^{NTR}$ has been implicated.

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## Footnotes

*Abbreviations used in this paper:*

p75$^{NTR}$-p75 low affinity neurotrophin receptor

FL-full length

DD-death domain

DR-death receptor

DN-dominant negative

ICD-intracellular domain

DED-death effector domain

NGF-Nerve Growth Factor

PI-propidium iodide

FADD-Fas-associated death domain protein

TRADD-tumor necrosis factor-associated death domain protein

TNFR-tumor necrosis factor receptor

vFLIP-viral flice inhibitory protein

DISC-death inducing signaling complex

## Figure Legends

FIG.1. **Diagram of human p75$^{NTR}$ constructs used in this study.** The amino acids at the ends of the domains and of the construct s are numbered.

FIG.2. **Expression of p75$^{NTR}$ in ST14A cells is cytotoxic.** *A*, P75$^{NTR}$ and TrkA mRNA expression were analyzed in ST14A cells. RT-PCR was performed on total RNA extracted from ST14A cells (lane 3 and 4) and cells stably transfected with p75$^{NTR}$ (lane 5-10). Lanes 5,6 and 9,10 are single clones while 7,8 are pooled p75$^{NTR}$ transfectants. Where indicated, ponasterone treatment was for 36 h. RT-PCR reactions were amplified for 15 or 30 cycles as indicated using the gene specific primers shown on the right. Negative controls (N. Ctrl.): SK-N-BE for p75$^{NTR}$; RNAase treated ST14A sample for TrkA. Positive controls (P. Ctrl.): Schwann cells for p75$^{NTR}$; A431 carcinoma for TrkA and GAPDH; GAPDH mRNA was selected as internal control. *B*, ST14A-p75$^{ind}$ cells were treated with ponasterone for 48 h at 33 °C. The transfectants were assayed at the indicated passage numbers. Viability of the cells was determined by the MTS assay and compared to controls that were treated identically except for the addition of ponasterone. Parental ST14A cells were included as the control for non-specific ponasterone effects. The values are mean ± S.E. of three determinations. *C*, Cell surface expression of FLAG-p75$^{NTR}$ in passage #2 and #40 cells was measured by FACS after overnight incubation with ponasterone.

**FIG. 3. P75$^{NTR}$ cytotoxicity is not modulated by NGF and requires the death domain.** *A*, ST14A-p75$^{ind}$ cells were treated with ponasterone for 48 h at 33 °C and the indicated culture conditions. Viability was measured with the MTS assay. Parental ST14A cells and transfectants expressing p75$^{NTR}$ deletions were assayed similarly. Mean values ± S.E. of three experiments are shown. Significant killing by ponasterone is indicated by *. *B*,

Expression of the FLAG tagged constructs was assayed by FLAG immunoprecipitation (I.P.) and Western blot (W.B.). For comparison with endogenously expressed p75$^{NTR}$, lysates containing equal amount of total protein were immunoprecipitated with α-p75$^{NTR}$ and analyzed on a Western blot with the same antibody. S.C. Rat Schwann cells, not treated. Arrows indicate p75$^{NTR}$ bands.

FIG.4. **Expression of p75$^{NTR}$ induces apoptosis.** *A*, The p75$^{NTR}$ transfectants were treated with ponasterone (Pon.) for the hours indicated. Total DNA was extracted from an equal number of cells, and separated on a agarose gel. *B*, Approximately 5 x $10^6$ ST14A-p75$^{ind}$ transfectants were left untreated or treated with 5 or 15 μM ponasterone for 48 h. All cells were harvested and homogenized. The ΔDD and ΔICD cell line was treated similarly and assayed for caspase-9 activity. Equal amounts of protein were incubated with 0.2 μM of Caspase substrates at 37 °C for a time course of 3 h. The fluorescence intensities were calibrated with free AFC and MCA. Substrate conversion rates were calculated using the slope of the time course and corrected by subtracting values obtained from the untreated extracts. The values are mean ± S.E. of three independent experiments. The inset shows the Caspase 8 control experiment: Cells were transiently transfected with 1μg pcDNA3.1, or 1μg TNFR1 for 24 h, or treated with 100 ng/ml TNFα plus 10 μM cycloheximide for 6 h. The lysates were assayed with the Caspase 8 substrate.

FIG.5. **General and DR specific inhibitors repress p75$^{NTR}$ induced apoptosis.** *A*, p75$^{NTR}$-FL stable lines were co-transfected with GFP expression vector and the plasmid expressing the indicated inhibitor. The cells were treated with ponasterone for 48 h and nuclei stained with PI. The PI staining pattern of transfected cells was evaluated by fluorescence

microscopy. The number of transfected cells with apoptotic PI staining over the number of total counted GFP positive cells is given. At least two independent experiments were evaluated. Significant inhibition compared to ponasterone treated control is indicated by * ($p<0.05$). *Inset)* Parental ST14A cells were transfected with TNFR1, GFP and TRADD DN and FADD DN constructs. Apoptotic DNA staining pattern was evaluated after 48 h as in A. *B*, p75$^{NTR}$-FL-FLAG stable lines were transfected similarly and processed for TUNEL assay. Transfected (GFP) and TUNEL positive cells were quantitated by FACS. As an example, the fluorescence intensity data from untreated (Ctrl.), vector control (Pon.) and E8 transfected cells is shown. The quantitation of the percentage of double positives is given. Results shown are from three separate experiments. Significant inhibition compared to ponasterone treated control is indicated by * ($p<0.05$).

FIG.6. **Dominant interference of a p75$^{NTR}$ mutant defective in apoptotic signaling.** *A*, p75$^{NTR}$-FL stable lines were co-transfected with GFP vector and the plasmids of mutants constructs or E8. Cells were treated with ponasterone for 72 h. Quantitation of apoptotic DNA condensation of the transfected cells was evaluated after PI staining. The results are from three experiments. Significant inhibition is indicated by * ($P<0.05$). Expression of p75$^{NTR}$ was monitored by FLAG immunoprecipitation and Western blotting. *B*, 293 cells were co-transfected with the indicated p75$^{NTR}$-FL-GFP construct and FLAG-tagged FL or mutant constructs. Lysates (Lys.) were immunoprecipitated (IP) and analyzed by GFP Western blot (W.B.). The gel was reprobed with FLAG to confirm expression of the FLAG-tagged constructs.

**Fig. 1**

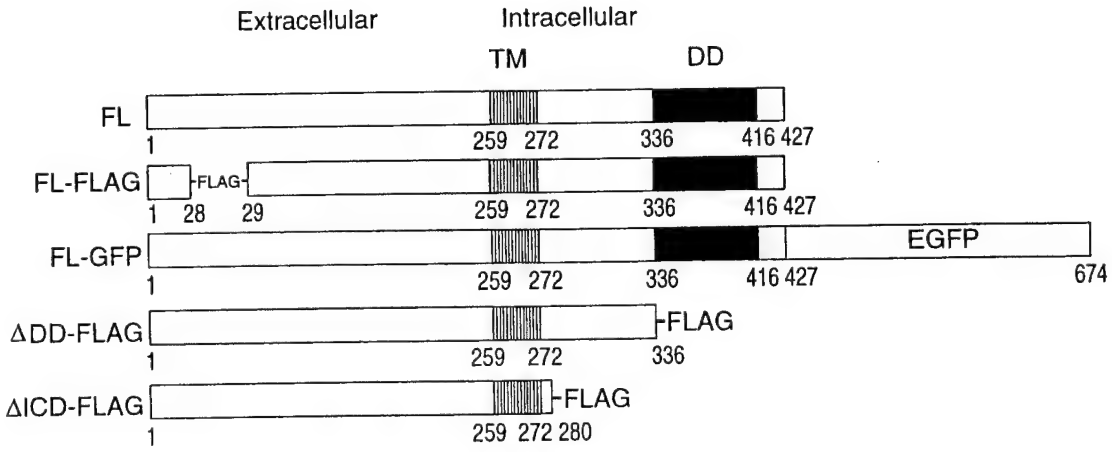
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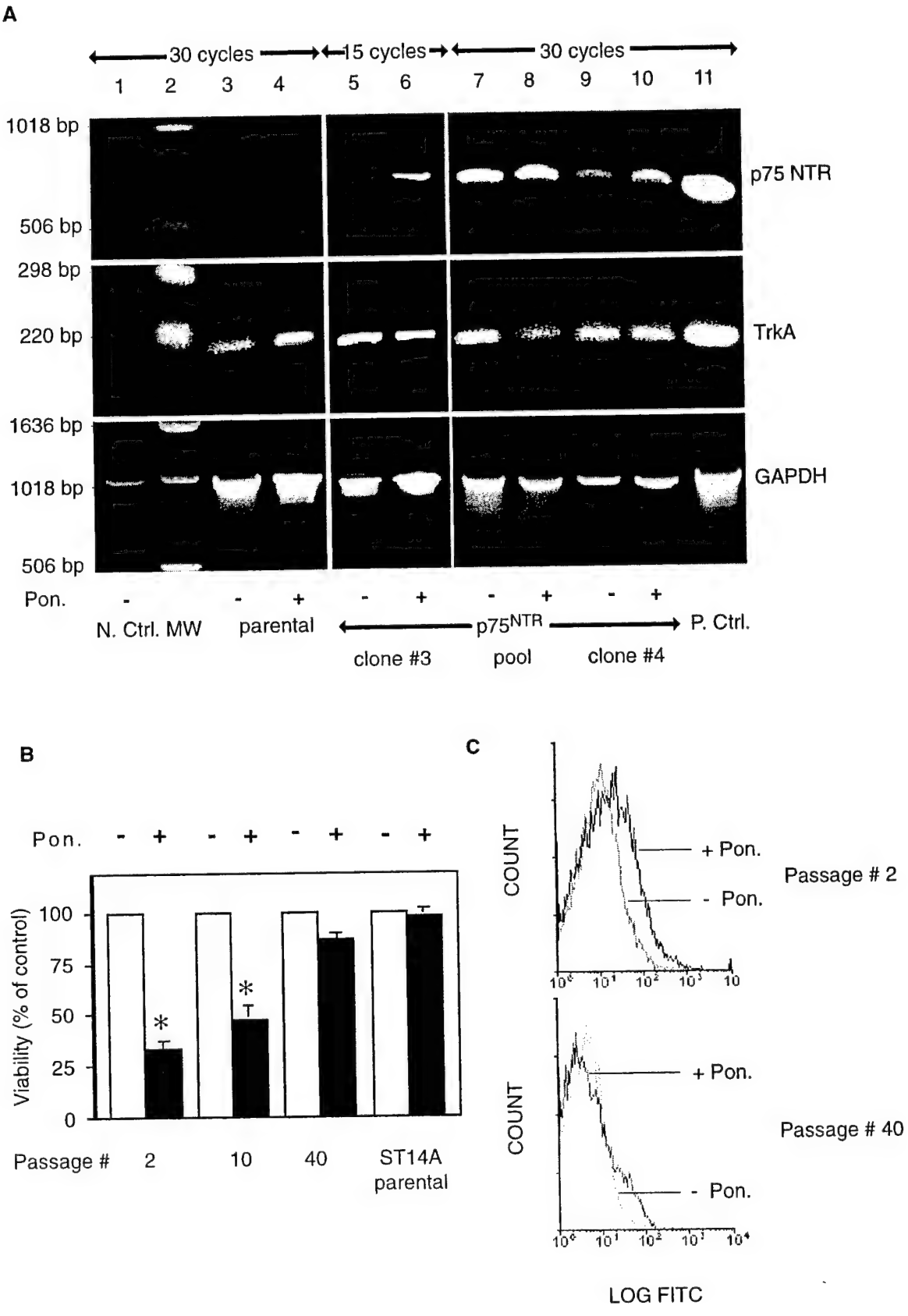
**Fig. 2**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30 cycles
15 cycles
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1018 bp
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p75 NTR
298 bp
220 bp
TrkA
1636 bp
1018 bp
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GAPDH
Pon. - - + - + - + - +
N. Ctrl. MW
parental
p75NTR
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clone #3
pool
clone #4
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Pon. - + - + - + - +
Viability (% of control)
100
75
50
25
0
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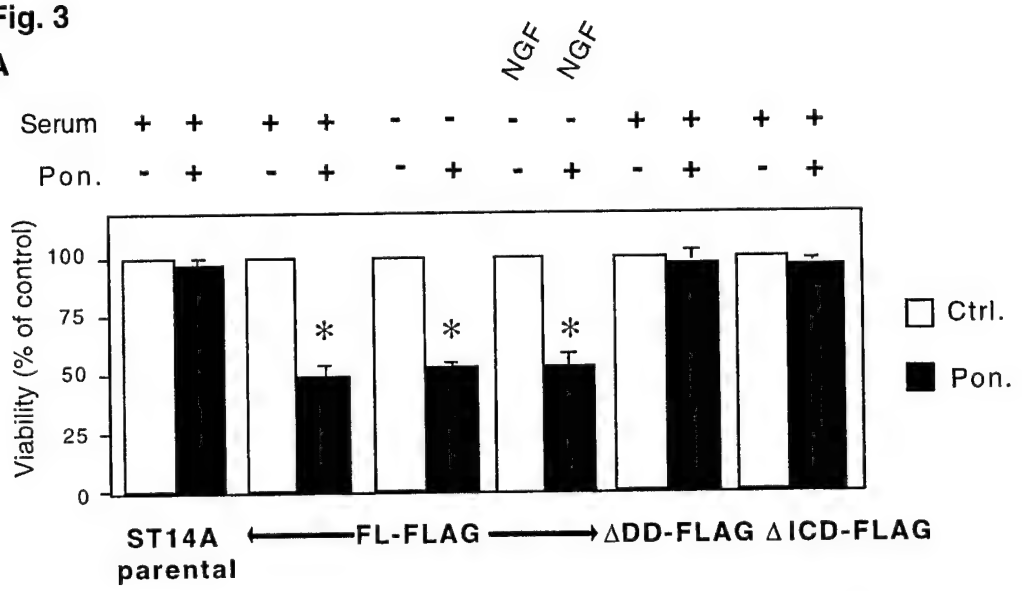
**Fig. 3**

**A**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Viability (% of control)
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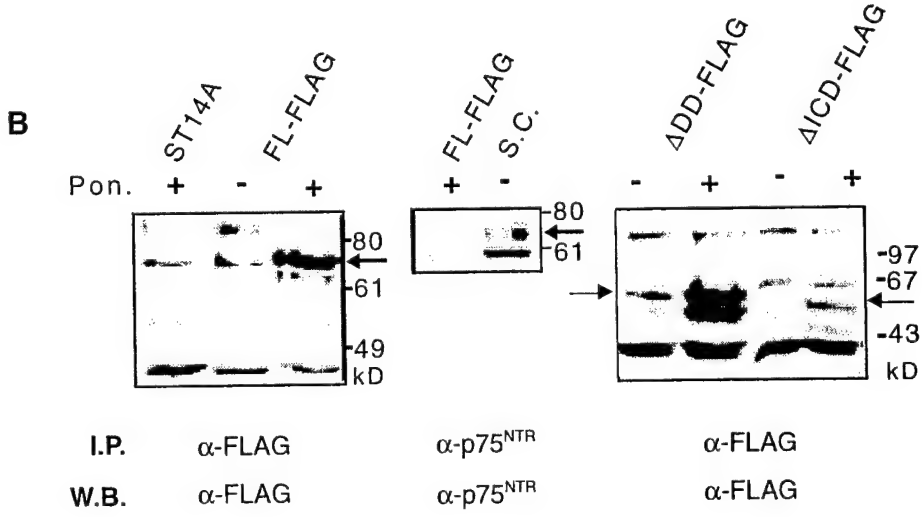
FL-FLAG
ΔDD-FLAG
ΔICD-FLAG

**B**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ΔICD-FLAG
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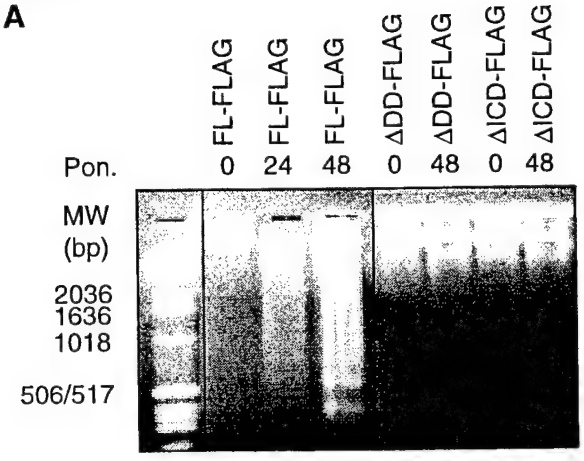
61
-97
-67
-43
kD
I.P. α-FLAG α-p75NTR α-FLAG
W.B. α-FLAG α-p75NTR α-FLAG

**Fig. 4**

**A**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FL-FLAG
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ΔDD-FLAG
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ΔICD-FLAG
ΔICD-FLAG
Pon.
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MW
(bp)
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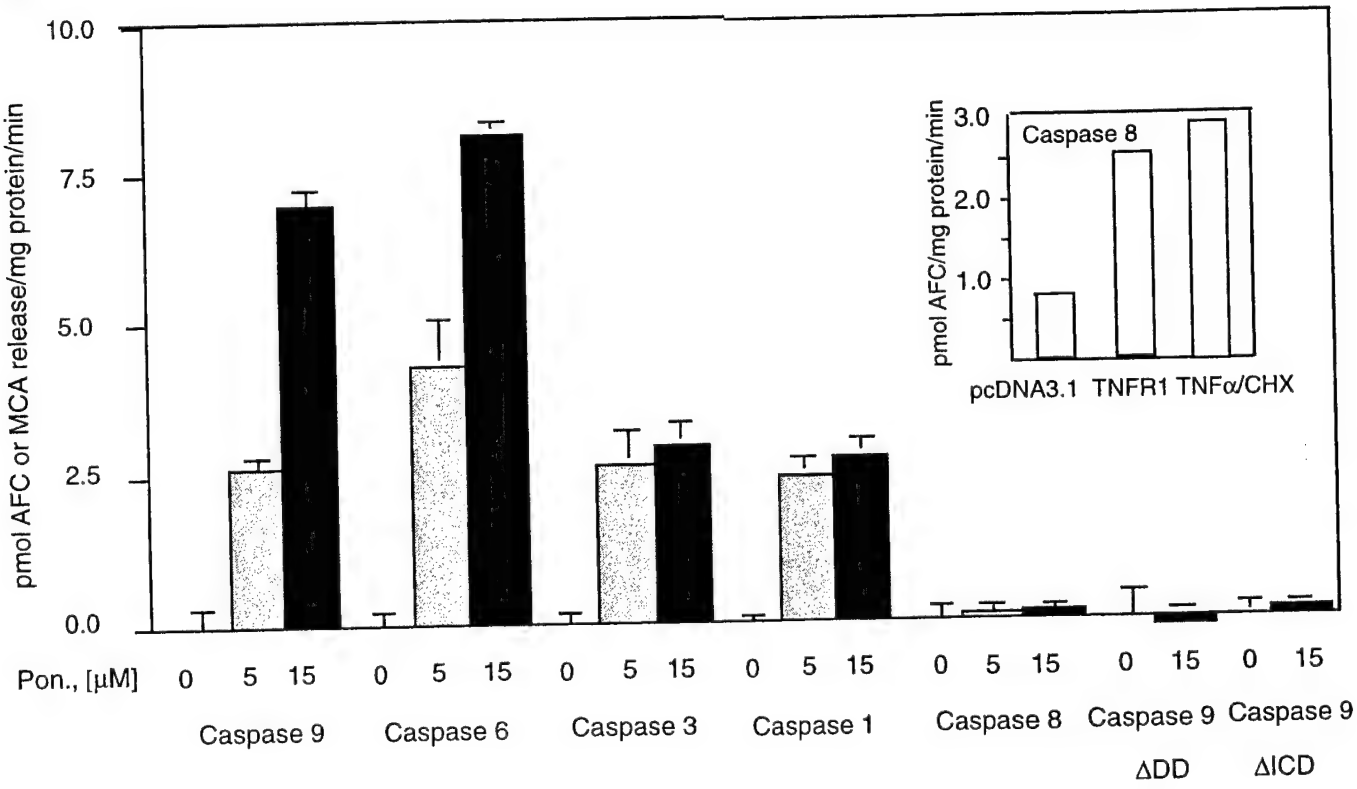
1636
1018
506/517

**Fig. 4**

**B**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pmol AFC or MCA release/mg protein/min
Pon., [μM] 0 5 15 0 5 15 0 5 15 0 5 15 0 5 15 0 15 0 15
Caspase 9
Caspase 6
Caspase 3
Caspase 1
Caspase 8
Caspase 9 ΔDD
Caspase 9 ΔICD
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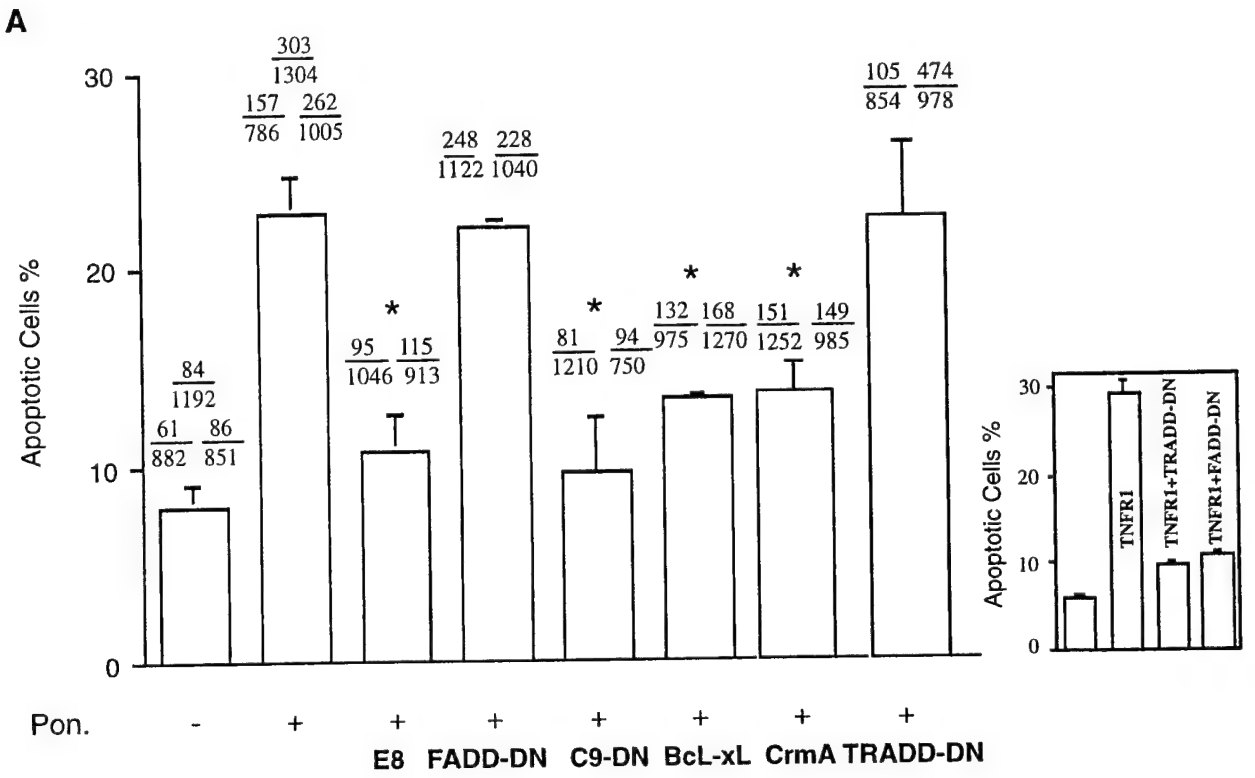
2.0
1.0
pmol AFC/mg protein/min
Caspase 8
pcDNA3.1 TNFR1 TNFα/CHX

Fig. 5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Apoptotic Cells %
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20
10
0
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61/882
86/851
303/1304
157/786
262/1005
95/1046
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248/1122
228/1040
81/1210
94/750
132/975
168/1270
151/1252
149/985
105/854
474/978
Pon. - + + + + + + +
E8 FADD-DN C9-DN BcL-xL CrmA TRADD-DN
Apoptotic Cells %
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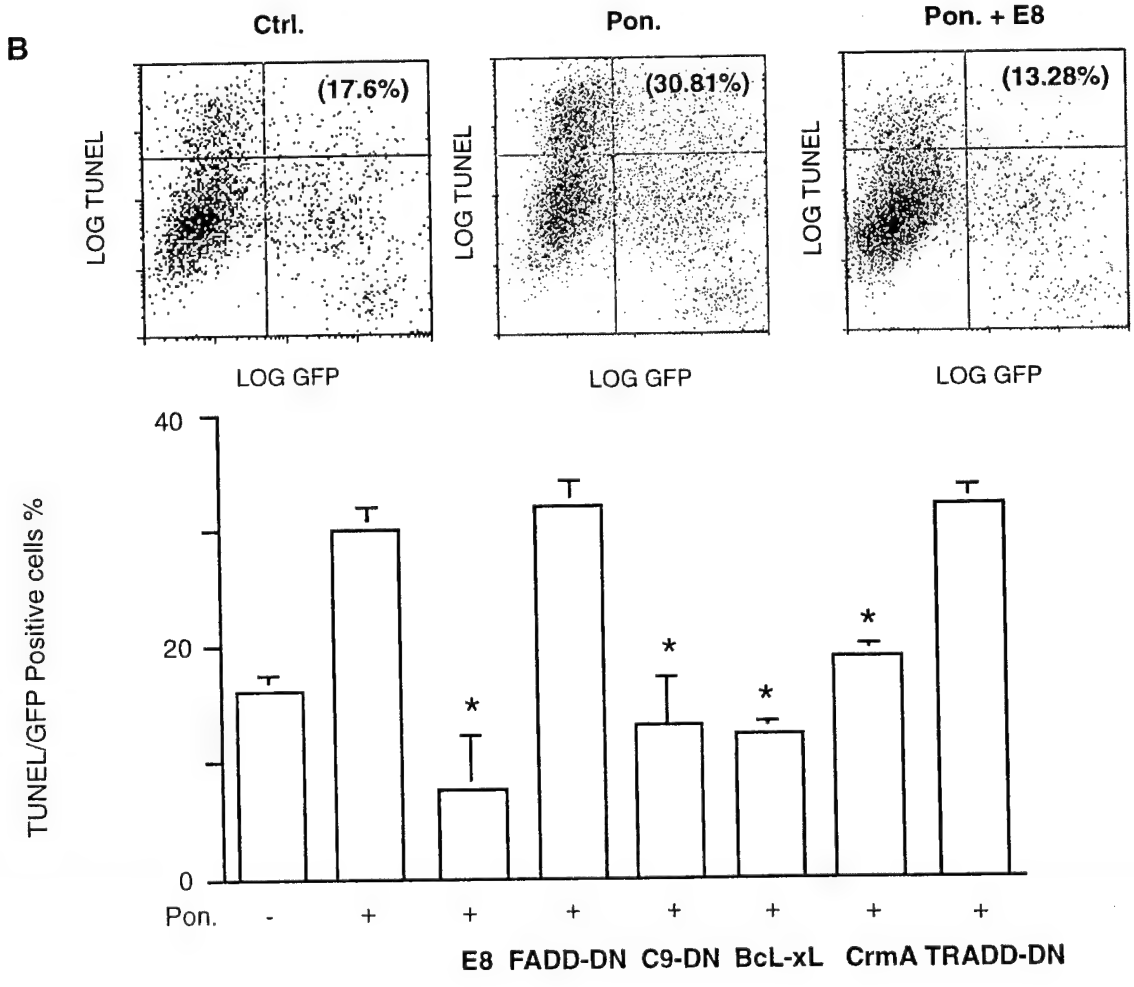
TNFR1
TNFR1+TRADD-DN
TNFR1+FADD-DN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Pon.
Pon. + E8
(17.6%)
(30.81%)
(13.28%)
LOG TUNEL
LOG GFP
TUNEL/GFP Positive cells %
40
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0
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E8 FADD-DN C9-DN BcL-xL CrmA TRADD-DN

# DEATH RECEPTORS AND APOPTOSIS

## Deadly Signaling and Evasive Tactics

Claudius Vincenz, PhD

The discovery in 1989 that monoclonal antibodies can recognize a specific protein on the surface of a lymphoblast cell line and induce apoptosis marks the beginning of the growth of the field of death receptors.[99] This antibody, αAPO-1, was able to induce apoptosis in a variety of transformed cell lines and, therefore, held promise for use in chemotherapy.

This article summarizes developments in the succeeding decade, including the addition of several new members to the death-receptor family and the elucidation of the signaling pathways involved. The emphasis of this article is to summarize these findings and juxtapose them to point out the similarities between the different receptors. The multiple signaling cascades emanating from most death receptors lead to complex biological responses. The goal of this article is not to discuss all those responses in detail, and the reader is referred to the pertinent reviews; however, the signaling components and their properties involved in the nonapoptotic responses are reviewed.

Death receptors have been a tempting pharmacological target even before their characterization at the molecular level and modest clinical successes were achieved by modulating the activity of death receptors. The discovery of tumor necrosis factor α [TNF-α]–related apoptosis inducing ligand (TRAIL), a new death receptor activating ligand however, lent new optimism for this approach.

Funding from DAMD17-96-6085 and NIH ES08111 is acknowledged.

### DEATH RECEPTORS: A SUBGROUP OF THE TUMOR NECROSIS FACTOR/ p75$^{NTR}$ FAMILY

Seven members of the death receptor family have been identified to date. Their names, ligands and, Genbank accession numbers are summarized in Table 1.

The denotation death receptors was given to this class of cell surface receptors because they all contain a death domain motif in their cytoplasmic portion. The death domain was originally identified in the Fas receptor.[48] The death domain itself is part of a structurally defined superfamily that includes the caspase recruitment domain (CARD), death effector domain (DED), and death domain (DD).[25, 29, 45, 86] Structurally, the death domain superfamily is characterized by an arrangement of six helices in a bundle. Functionally, these domains mediate homotypic interactions and are found as part of death receptors, adapter mol-

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan

**Table 1.** DEATH RECEPTORS AND THEIR LIGANDS

| Name | Alternate Names | Ligand | Genbank Accession cDNA | | Genbank Accession Protein | |
|---|---|---|---|---|---|---|
| | | | *Human* | *Mouse* | *Human* | *Mouse* |
| *Death Receptors* | | | | | | |
| TNFR-1 | p55TNFR, CD120a | TNF | M33294 | L26349 | M75866 | P25118 |
| Fas | Apo-1, CD95 | FasL | X63717 | NM_007987 | P25445 | NP_032013 |
| DR3 | Apo-3, LARD, TRAMP, Wsl-1 | Tweak | NM_003790 | | NM_003790 | |
| DR4 | Apo-2, TRAIL-R1 | TRAIL | U90875 | | NP_003835 | |
| DR5 | TRAIL-R2, TRICK2 | TRAIL | AF012535 | AF176833 | AAB67103 | AF176833 |
| DR6 | — | ? | AF068868 | | AAC34583 | |
| $p75^{NTR}$ | | NGF, BDNF, NT-3, NT-4/5, NT-6 | NM_002507 | AF105292 | PO8138 | AAD17943 |
| *Decoy Receptors* | | | | | | |
| DcR1 | TDRIDD | TRAIL | AF012629 | | AAB67104 | |
| DcR2 | TRAIL R4, TRUNDD | TRAIL | AF021232 | | NP_003831 | |
| DcR3 | — | FasL | AF104419 | | NP_003814 | |

TNFR-1 = Tumor necrosis factor receptor 1; DR = death receptor; DcR = decoy receptor; $p75^{NTR}$ = low affinity neurotrophin receptor; NGFR = nerve growth factor receptor; BDNF = brain derived neurotrophic factor; NT = neurotrophin; TNF-α = tumor necrosis factor α.

ecules, and kinases. Death domains mediate the binding of death domain–containing adapters to the cytoplasmic tails of the death receptor. This interaction is initiated by the trimerization of the receptors on ligand binding.

Other sequence motifs are not conserved in the cytoplasmic tails of the death receptors. The remainder of the cytoplasmic tails can be very short, as in Fas, or contain multiple potential signaling motifs, as in DR6.

The extracellular domains of the death receptors all contain the cysteine rich repeats, which define the superfamily of tumor necrosis factor receptor (TNFR)/nerve growth factor receptor (NGFR). All ligands for the receptors belong to the TNF ligand family. The notable exception is $p75^{NTR}$, which binds with low affinity to neurotrophins (NGF, brain derived neurotrophic factor [BDNF], neurotrophin [NT]-3, NT-4/5, NT6) which are not related to TNF-α. Organization of the domains on death receptors is summarized in Figure 1.

## APOPTOTIC SIGNALING FROM DEATH RECEPTORS

Killing by death receptors on ligand binding is masked in most instances by the ability of these receptors to simultaneously induce protective and suicidal responses. In addition, numerous studies indicate the presence of death receptor specific antiapoptotic proteins in some cell types and tissues. Therefore, expression of a death receptor does not necessarily render all cells susceptible to killing by the cognate ligand.

### Fas

The signaling from the Fas receptor is the least complex because it generates predominantly a proapoptotic signal. On Fas ligand (FasL) binding, the receptor is trimerized, which leads to the recruitment of the adapter molecule Fas-associated death domain (FADD) to the receptor complex. FADD originally was identified through a yeast two hybrid screen as a Fas binding protein.[12, 23] The interaction of FADD with Fas is mediated through their mutual death domains. Further transmission of the death signal is achieved by recruitment of caspase-8 to the complex. This reaction is mediated by homotypic interactions between the DEDs of FADD and the prodomain of caspase-8.[11, 75] Caspases are aspartate specific proteases, which are produced as inactive zymogens and require activation by proteolytic processing.[79] Once recruited, caspase-8 can proteolytically process itself, which leads to its full activation.[71, 76, 110] Because caspases can activate each other, the

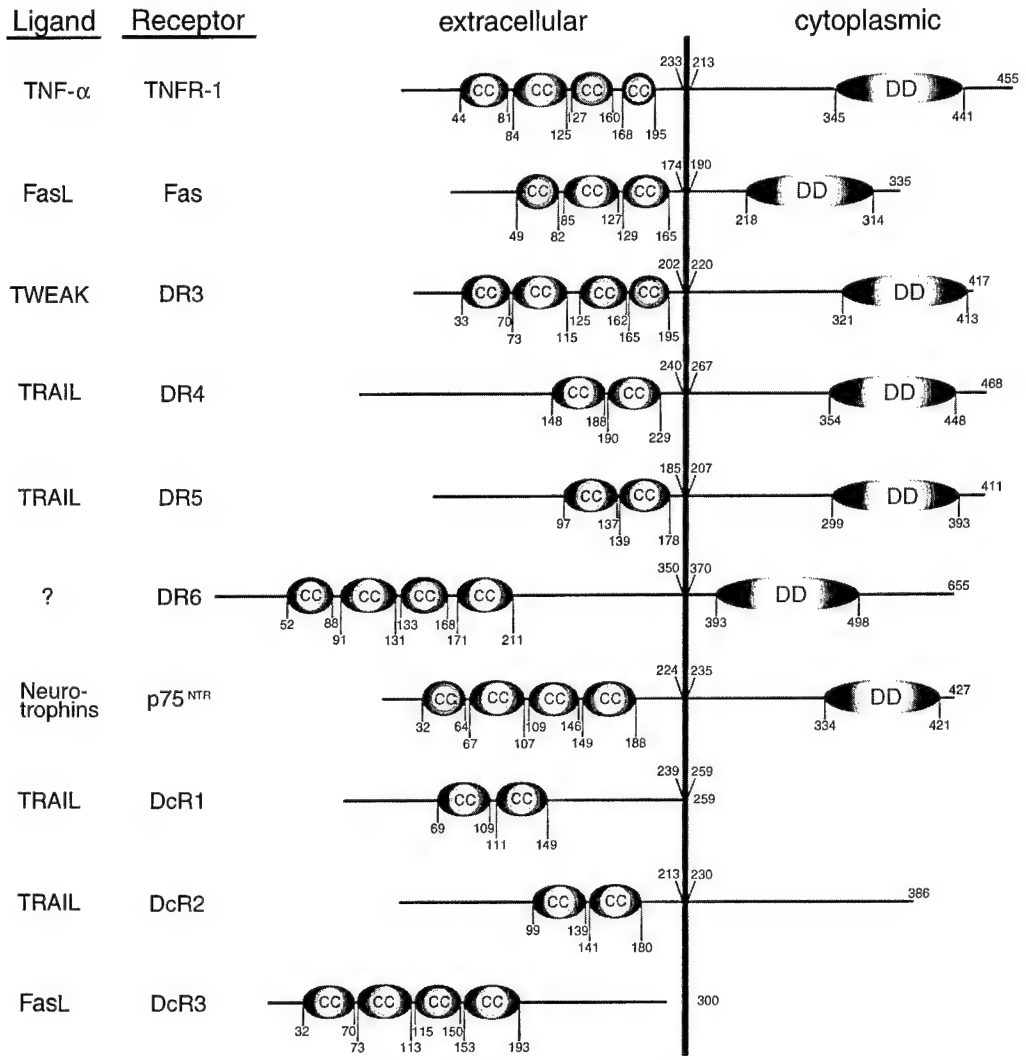


**Figure 1.** Domain organization of death receptors. DcR = Decoy receptor; cc = cysteine rich repeats; DD = death domain; DR = death receptor; FasL = Fas ligand; $p75^{NTR}$ = low affinity neurotrophin receptor; TNF-α = tumor necrosis factor α; TNFR-1 = TNF receptor 1; TRAIL = TNF-α–related apoptosis inducing ligand.

generation of active caspase-8 triggers the activation of a caspase cascade, including the more abundant downstream caspase-3, -6 and -7. The caspases themselves cleave multiple proteins critical for cellular function producing the orderly disassembly of the cell. This suprisingly direct pathway from Fas receptor ligation to protease activation provides the molecular framework from which one can understand apoptotic signaling from all other death receptors.

In some cell types, caspase-8 activation alone is not sufficient to kill cells, and the death signal needs to be amplified.[84] This is achieved by activation of the death machinery that is dependent on the release of cytochrome c from mitochondria.[66] Cytochrome c associates and activates the apoptosome complex consisting of apoptotic protease-activating factor 1 (Apaf-1) and caspase-9, which then activates the downstream Caspase-3, -6, and -7.[14, 118] The link between caspase-8 and the mitochondrial pathway is Bid.[65, 68] This proapoptotic Bcl-2 homologue is cleaved by caspase-8 and cleaved Bid initiates the release of cytochrome c from mitochondria. The importance of this pathway is best revealed in the Bid knock out mice. These mice are more

resistant to injection of agonistic Fas antibody. This treatment leads to rapid death by liver destruction in wild type littermates.[114]

### Tumor Necrosis Factor Receptor 1

The apoptotic signaling form the TNF receptor 1 (TNFR-1) (p55) is analogous to Fas signaling. The first evidence for this similarity was the activity of a FADD dominant negative (DN) construct which inhibited not only Fas but also TNF signaling.[21] Subsequent studies with FADD and caspase-8$^{-/-}$ mice revealed that these animals had a complete defect in Fas and TNF signaling, clearly establishing the role of these molecules in these pathways[100, 112]; however, TNFR-1 death signaling requires an additional upstream adapter, TNFR-associated death domain protein (TRADD), which was identified by a yeast two hybrid screen using TNFR-1 as bait.[42] TRADD is a death domain containing protein that recruits FADD to the death receptor connecting the TNFR-1 death signal to the Fas pathway. TRADD can also recruit TNFR-associated factor 2 (TRAF2) and RIP to the receptor complex. TRAF2 and RIP mediate nonapoptotic signals that are discussed later.

### DR3

The signaling from death receptor 3 (DR3) is similar to TNFR-1.[9, 22] It uses TRADD and FADD as adapter molecules, and caspase-8 is the apical caspase activated by the receptor complex.

### DR4/DR5

DR4 and DR5 are homologous in structure and bind the ligand TRAIL. Partially conflicting reports have been published about the nature of the adapter molecule. Experiments with FADD$^{-/-}$ embryonic fibroblasts transfected with DR4 showed that apoptosis was not affected.[112] Experiments in cellular systems have revealed an inhibitory effect by FADD DN.[44, 101] Recently it was shown that endogenous FADD associates with activated endogenous DR4/DR5. FADD is also needed for DR5-induced apoptosis in a cellular system, providing strong evidence that FADD is the adapter protein for DR5 killing.[10]

More controversial is the involvement of TRADD in TRAIL signaling. Coimmunoprecipitation experiments have indicated some affinity of TRADD for DR4/DR5.[19, 91] The TRADD DN had no effect on TRAIL function, however.[44]

The nature of the apical caspase in DR4/DR5 is not yet firmly established. Interactions measured in overexpression experiments have identified caspase-8 and -10 as likely candidates.[82] A potential involvement of caspase-10 is suggested by the observation that dendritic cells from patients carrying caspase-10 mutations have a defect in TRAIL signaling[105]; however, careful analysis of the endogenous caspase recruited to the TRAIL receptors reveal that only caspase-8, and not Caspase-10, is recruited and processed by DR4/DR5 receptor activation.[10] A potential explanation for these contradictory results is that mutant caspase-10 behaves as a nonreleasable substrate, thereby inhibiting caspase-8 activation. This would be analogous to the naturally occurring inhibitor viral FLICE inhibitory protein (vFLIP), which inhibits caspase-8 activation by Fas and TRAIL.[97] Confirmation of this hypothesis must await the production of caspase-10$^{-/-}$ mice.

### DR6

Very little is known about the mechanisms that control death signaling from DR6. In contrast to death receptors TNF, Fas, and DR3-5, overexpression of DR6 only kills HeLa (cervical carcinoma) but not MCF7 (breast cancer) cell lines.[83] The only adapter molecule that has an affinity for DR6 is TRADD; however, this association has yet to be shown to occur with the endogenous molecules.

### p75$^{NTR}$

The founding member of the superfamily of TNF/p75$^{NTR}$-receptors is p75$^{NTR}$.[53] Many fundamental questions about p75$^{NTR}$ function and signaling remain unanswered. Because the p75$^{NTR}$ ligand, the neurotrophin NGF, induces cell survival and differentiation in most systems, p75$^{NTR}$ was not considered a genuine

death receptor; however, data generated in systems that express only $p75^{NTR}$ and not the high affinity NGF receptor, tyrosine receptor kinase (TrkA), established that $p75^{NTR}$ can induce apoptosis.[16, 33] Further confirmation that $p75^{NTR}$ is a death receptor was obtained by the multiple analyses performed on $p75^{NTR-/-}$ mice.[17]

The mechanism of $p75^{NTR}$-induced cell death is poorly understood. Recent studies have established convincingly that $p75^{NTR}$-induced death proceeds by an apoptotic mechanism because it is inhibited by Bcl-xL and caspase inhibitors.[27, 37] A chimaeric receptor consisting of the extracellular domain of Fas and the intracellular domain (ICD) of $p75^{NTR}$ did not induce apoptosis in cells susceptible to Fas killing.[58] Therefore, intracellular signaling of $p75^{NTR}$ is likely to involve different molecules than those that are known to mediate Fas signaling. Studies with transgenic mice demonstrated the involvement of the intracellular domain of $p75^{NTR}$ in apoptotic signaling.[69] Surprisingly, it has been reported that the death domain of $p75^{NTR}$ is not needed to generate an apoptotic signal[27]; however, the conserved death domains mediate the apoptotic signal from all other death receptors and mutational studies performed in neuronal cellular systems show that it is also necessary for $p75^{NTR}$ death signaling (CV, unpublished data, 2000). Studies using yeast two hybrid screens have identified NRIF, SC-1, and the GTPase RhoA as candidate molecules that directly communicate with the cytoplasmic domain of $p75^{NTR}$.[18, 24, 109] Further studies will reveal if these molecules mediate apoptotic or other $p75^{NTR}$ signals (Fig. 2).

## NONAPOPTOTIC SIGNALING FROM DEATH RECEPTORS

Early experiments revealed that cells normally resistant to the cytotoxic effects of TNF-α could be sensitized by using cycloheximide, which is an inhibitor of protein synthesis. In addition, interleukin (IL)-1 treatment, an inducer of nuclear factor κB (NF-κB), increased the resistance to TNF-α.[41] The same observa-

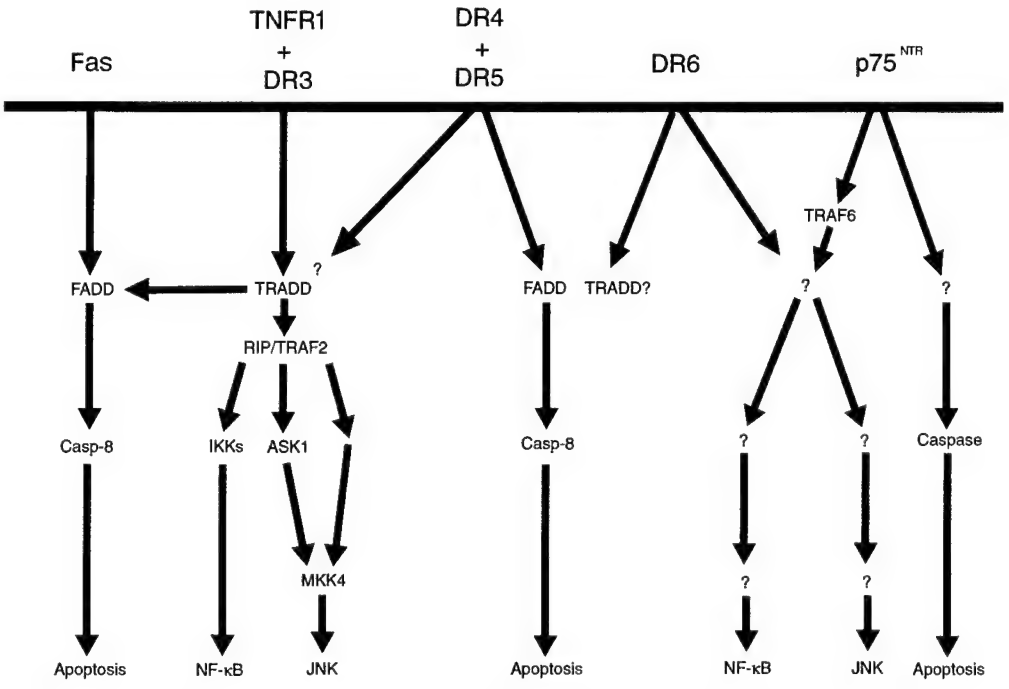


**Figure 2.** Signaling molecules for death receptors. Casp-8 = Caspase-8; DR = death receptor; FADD = Fas-associated death domain; IKKs = Iκ B kinase; JNK = Jun kinase; NF-κB = nuclear factor κB; TNFR-1 = tumor necrosis factor receptor 1; TRADD = TNFR-associated death domain protein; TRAF = TNFR associated factor; $p75^{NTR}$ = low affinity neurotrophin receptor.

tion were made more recently using specific inhibition of NF-κB. Expression of inhibitor of κB (IκB) DN inhibits NF-κB activation effectively. In agreement with the earlier data, a drastic sensitization to TNF-α–induced apoptosis was observed on inhibition of the NF-κB pathway.[5] Therefore, activation of NF-κB is in most instances results in a prosurvival or antiapoptotic response.

## Tumor Necrosis Factor Receptor 1

Mechanistic insight into the TNF-R1–activated NF-κB pathway was obtained by yeast two hybrid screens and biochemical purification of receptor associated proteins. TRADD serves a dual function and activates NF-κB and apoptosis.[42] RIP, a kinase with a death domain, interacts with TRADD and is also able to induce NF-κB and apoptosis[43]; however, RIP plays a predominant role in NF-κB signaling because the $RIP^{-/-}$ mice have a defect in NF-κB signaling and are sensitized rather than resistant to TNF-α–induced apoptosis.[56] A third group of proteins involved in the nonapoptotic responses of TNF-R1 is the TRAF family of proteins.[88] TRAF proteins can also associate with the TNFR-1 signaling complex. Of the six family members, TRAF1 and TRAF2 have been implicated in signaling from TNFR-1. The $TRAF2^{-/-}$ mice only have a defect in TNFR-1 induced Jun kinase (JNK) activation[113]; however, NF-κB activation might be mediated by multiple redundant TRAFs. Upcoming studies on double knock out mice will resolve any ambiguity. The NF-κB signal transduction from TRAF and RIP to the IκB kinase complex is not fully understood. NF-κB inducing kinase (NIK) and MEK kinase 1 (MEKK1), members of the mitogen activated protein kinase kinase kinase (MAPKKK) family, have been proposed to mediate this step. According to this model, a kinase cascade is activated that leads to phosphorylation and activation of IκB kinase α (IKKα) and/or IKKβ.[55] Alternatively, IKKs could be auto-activated by RIP-mediated aggregation, eliminating the need for a kinase cascade.[117] The signaling pathway downstream of the IκB kinase complex is identical for the TNFR-1 or other members of the superfamily and many other stimuli that can activate NF-κB. Discussion of these common signaling pathways is beyond the present scope but is available in recent reviews.[36, 115]

TNFR-1 also induces the JNK and p38 pathways. The bifurcation point between NF-κB and JNK is at the level of the TRAF molecules.[94] Probably multiple kinases can transmit the signal to the MAPKK that activates JNK. ASK1 has been identified as one of the kinases that is activated by TRAFs and activates the JNK pathway.[40, 78] Activation of these pathways contribute to the complex pleiotropic effects of TNF-α which are summarized elsewhere.[49, 103] JNK activation by TNF-α does not lead to an apoptotic response because this activation is only transient and continued JNK activation is required for a death response by way of this pathway.[67, 74]

TNFR-1 also activates the ERK pathway, which seems to contribute to the mitotic effects of TNF-α.[103] The TNFR-1–associated protein, MADD, has been implicated in this pathway.[90] It is not known how the ERK activating signal is transduced from the receptor complex to the kinase cascade that activates ERK.

## DR3

Much less is known about nonapoptotic signaling from DR3 but it is likely to be homologous to TNFR-1. The death domain of DR3 is almost identical to TNFR-1 and associates with TRADD, which leads to activation of the death and NF-κB pathways.[9, 22, 70] Overexpression experiments reveal that TRAFs have the same role in activation of the NF-κB pathway by DR3 as they have for TNFR-1.

## Fas

Multiple studies have shown that Fas ligation activates the JNK pathway.[62] Several reports indicate that JNK activation is only a secondary event because it is abrogated by caspase inhibitors[13, 54, 64]; however, caspase independent activation of the JNK pathway has also been demonstrated, and the discovery of the adapter protein DAXX even suggests a plausible mechanism.[98, 111] Nevertheless, the complete defect in Fas-induced apoptosis in the $FADD^{-/-}$ mice indicates that JNK activa-

tion plays no role in Fas-induced apoptosis.[112, 116]

### DR4/DR5

TRAIL receptor activation induces NF-κB.[44] Overexpression experiments have indicated that TRADD could be the adapter molecule, but data on interactions between the endogenous molecules or on TRADD$^{-/-}$ mice are still lacking.[91] Despite these similarities to the TNFR-1 signaling, the effect of NF-κB activation is very different on TRAIL- versus TNFR-1–induced apoptosis. Shutdown of the NF-κB pathway by expression of the dominant negative form of IκBα leads to sensitization to TNF-induced apoptosis. Expression of this mutant IκBα has no effect on TRAIL-induced apoptosis.[44] Further, NF-κB induction occurs at lower doses of TNF-α than apoptosis. In contrast, TRAIL-induced NF-κB activation and TRAIL-induced cell death occurs with similar dose dependency (H. Wajant, personal communication, Dr. Stuttgart, Germany, 2000); however, some similarities with respect to inhibition of their death responses between TNFR-1 and TRAIL receptors do exist. Treatment of cells with IL-1 also leads to protection of transformed keratinocytes from TRAIL-induced cytotoxicity.[60]

The JNK pathway is also activated by the TRAIL receptors.[44, 74] A sustained JNK activation lasting longer than 5 hours is observed. Depending on the cell type, this activation can be caspase dependent or independent.[74] Although the full physiological effect of this JNK activation is not known, it is apparent that it does not affect the apoptotic response.[44]

### DR6

Overexpression of DR6 causes activation of NF-κB and JNK. Information is not available yet on physiological responses nor on the the signaling mechanism.[83]

### p75$^{NTR}$

Multiple signals are also generated by the p75$^{NTR}$. Interestingly, only the ligand NGF is able to induce NF-κB even though all neurotrophins bind to p75$^{NTR}$.[15] TRAF6 has been shown to interact with the receptor in a signal dependent manner.[57] Experiments in Schwannoma cell lines suggest that inhibition of the pathway by dominant negative IκBα results in sensitization of the cells to NGF-mediated apoptosis.[35] It has yet to be determined whether p75$^{NTR}$-mediated NF-κB activation contributes to the survival of neurons and glia. Systems will have to be chosen where the high affinity TrkA receptor does not mask the p75$^{NTR}$ signal.

In analogy to the other death receptors, p75$^{NTR}$ also activates the JNK pathway.[4, 35] A close correlation between phosphorylation of the transcription factor JUN and induction of apoptosis is observed in these studies, but it has yet to be established whether p75$^{NTR}$ induction of JNK is sufficient to cause apoptosis.

## INHIBITORS OF DEATH RECEPTOR SIGNALING

### Inhibition of Death Receptor Autoactivation

As death receptors often are constitutively expressed, mechanisms must exist to guard against autoactivation of this lethal weapon. The silencer of death domain (SODD) functions in this manner and inhibits recruitment of TRADD and TRAF2 to the TNFR-1 and DR3.[52] Similar SODD-like inhibitors are expected to exist for the other death receptors although the homologous proteins have not yet been identified.

### Decoy Receptors

Some cell types protect themselves from the lethal effects of ligand binding by displaying decoy receptors on the cell surface (see Table 1). These receptors have intact extracellular ligand binding domains but do not contain a functional death domain. The TRAIL decoy receptors, DcR1 and DcR2, are the two receptors belonging to this class.[3] The initial hy-

pothesis that decoy receptors simply act by scavenging the ligand is an oversimplification.[82, 92] The inhibition by DcR1 is only partial and transient while DcR2 is more effective.[28] Because DcR2, in contrast to DcR1, retains a substantial portion of the cytoplasmic domain and is capable of activating NF-κB, the protection may be through scavenging intracellular signaling components or activation of an antiapoptotic signaling cascade.

DcR3 is secreted and binds FasL. It has been implicated in immune-evasion by colon and lung cancers.[85] Osteoprotegrin is also a secreted TNFR-1 homologue that can bind TRAIL, in addition to its cognate ligand osteoprotein ligand (OPGL)/RANKL, and could therefore function as a decoy receptor.[31]

### Fas-Associated Death Domain–Like Interleukin-1β Converting Enzyme–Like Inhibitory Proteins

Inhibition of the death signal can also be achieved after receptor stimulation. Cellular Fas-associated death domain–like interleukin-1β converting enzyme (FLICE)–like inhibitory proteins (cFLIP) prevent the aggregation and activation of caspase-8 and -10.[47] Viruses have adapted this strategy to specifically inhibit death receptor signaling by expressing vFLIP.[97] E8 and MC159 are examples of vFLIPs produced by equine herpes virus 2 (EHV-2) and molluscum contagiosum virus (MCV), respectively. The evolutionary pressure that led to the emergence of these PAN-death receptor inhibitors highlights the importance of this class of molecules in the host immune defense. It also suggests that death receptor signaling needs specialized inhibitors as many viruses express proteins homologous to Bcl-2 in addition to the vFLIPs.

### Bcl-2 Homologues

Some reports suggest that the class of Bcl-2 homologues, which are general inhibitors of apoptosis, can also inhibit death receptors. Bcl-2 and its homologues inhibit Fas agonistic antibody-induced cell death only in certain cell types.[89] Bcl-2 is ineffective if cell death is induced by the natural ligand, FasL.[46] It is not clear if this difference is merely because the intensity of the signal generated by the agonistic antibody and the FasL, respectively, or if the nature of the signal is different. Studies with TNFR-1 and $p75^{NTR}$ indicate that Bcl-2 homologues do affect death receptor function. Bcl-xL, a Bcl-2 homologue, has been shown to protect from TNF-α–and NGF-induced apoptosis.[27, 50, 96] Studies in multiple myeloma cells, however, show that Bcl-2 expression does not affect sensitivity toward TNF-α and TRAIL.[34] In sum, several reports suggest that Bcl-2 homologues inhibit death receptor signaling at least in circumstances where high Bcl-2 expression is combined with a weak or nonnatural death-receptor stimulus.

### Nuclear Factor κB

As outlined above, most death receptors induce protective responses and these are predominantly mediated by the NF-κB pathway. A series of gene products have been shown to be antiapoptotic and inducible by TNF-α. These include MnSOD, plasminogen activator inhibitor, cIAP2, TRAF1, IEX-1L, and A20.[61, 81, 104, 107–108] Because TNFR-1 is an effective activator of NF-κB, the protective effects of these genes have been mainly documented from TNF-α. Given the similarities in death receptor signal transduction, it is likely that some of these molecules inhibit multiple death receptors. A comprehensive review of the role of NF-κB in apoptosis was recently published by Barkett and Gilmore.[6, 32]

### Death Receptor Specific Inhibitors

Among the inhibitors induced by NF-κB, A20 stands out on account of its unique ability to inhibit apoptosis and the NF-κB pathway.[51, 81, 95] A20 is also an example of a specific inhibitor that only effects certain death receptors (TNFR-1) and does not inhibit others (Fas).[106]

Lifeguard (LFG) is another example of an inhibitor with high specificity. In contrast to A20, it is constitutively expressed in the brain and inhibits Fas but not TNF-α–induced

apoptosis.[93] LFG is a member of the seven membrane spanning protein G coupled receptors. How proteins of this class might specifically interfere with Fas signaling is not understood. Quite likely, additional death receptor specific inhibitors will be identified using the powerful functional cloning used for LFG.

Tissue specific expression of the inhibitor is another way to achieve selectivity as exemplified by ARC, which is only expressed in skeletal and myocardial muscle tissue.[59] Overexpression of ARC inhibits Fas and TNFR-1–induced apoptosis, it also inhibits hypoxia-induced apoptosis in embryonic heart-derived H9c2 cells. The precise mechanism of ARC inhibition remains to be elucidated, but it interferes with steps before cytochrome c release from the mitochondria.[30]

Control of cellular localization also has been shown to be an effective mechanism for regulating death receptor function. p53-induced apoptosis can be dependent of Fas signaling. It has been shown that p53 induces apoptosis by transiently increasing transport of Fas from the Golgi complex to the cell surface and sensitizing the cells to Fas.[7]

## THE PHYSIOLOGICAL ROLE OF DEATH RECEPTORS

Most death receptors generate multiple signals leading to a variety of biological responses. This pleiotropic aspect of death receptor signaling is best documented for TNF-α. TNFR-1$^{-/-}$ mice have deficient defenses against certain intracellular pathogens and a compromised immune response.[84, 87] Other recent reviews can be consulted for a more complete description of all the effects of TNF-α.[49, 103]

The naturally occurring *lpr* and *gld* mice have defects in Fas and FasL, respectively.[77] These mice suffer from lymphoproliferative disease manifesting itself in the expansion of lymphoid organs implicating Fas and FasL in lymphoid survival.

To date gene targeting experiments have not been reported on TRAIL and their receptors. Therefore, the physiological role of this ligand receptor system remains poorly defined; however, such information would be of great interest for estimating the potential side effects in settings in which TRAIL is used as a cancer therapeutic agent.[102]

Many detailed analyses have been performed on the p75$^{NTR-/-}$ mice.[17] The main deficit is in the peripheral nervous system.[63] Sympathetic, trigeminal, and cholinergic forebrain neurons are also affected. In accordance with a proapoptotic role for p75$^{NTR}$, increased number of neurons or neurons that die more slowly are found in p75$^{NTR-/-}$ mice; however, activation of p75$^{NTR}$ can also lead to increased cell migration and enhance the prosurvival effects of TrkA.[1, 8]

## DEATH RECEPTORS AS PHARMACOLOGICAL TARGETS

The potential to use activation of death receptors to combat malignancies predates the characterization of these proteins at a molecular level.[26] Experiments with injection of recombinant TNF-α into mice carrying a tumor burden revealed that the tumoricidal activity was caused by destruction of the vasculature of the tumors rather than because of the destruction of the tumor itself.[39] Consequently the effective doses are also often lethal. This severe toxicity is also a problem for approaches that aim at inhibiting the NF-κB pathway to down-regulate the inflammatory response. Severe TNFR-1–mediated liver toxicity results if NF-κB is inhibited throughout development.[72] This toxicity is less severe in adults but could still pose a problem for such an approach.[62] Similarly, injection of recombinant FasL or agonistic Fas antibody causes death within hours owing to liver toxicity.[80]

A more promising approach is the administration of recombinant TRAIL. Doses that are effective for tumor regression are tolerated well by mice.[102] First results using TRAIL injections as a sensitizer for ionizing radiation during the treatment of breast cancer are also encouraging.[20]

Trials with recombinant NGF also have been performed to ameliorate neuropathies associated with HIV and diabetes.[2, 73] This approach takes advantage of the prosurvival function of this receptor. It is possible that some of the problems encountered during

these clinical trials, including pain at the injection site, are owing to the proapoptotic activities mediated by NGF through $p75^{NTR}$.

## SUMMARY

The availability of large amounts of sequence data has made it possible to identify death receptors by homology. Because the genome has not been analyzed completely, a few additional members of this family probably will be identified in the next few years.

Rapid progress also has been made recently on the signaling mechanisms used by the death receptors. Considerable conservation of the intracellular signaling mechanisms is seen between different receptors suggesting that it is unlikely that new elements will be added to the molecular framework of death receptor signaling.

The analysis of signaling mechanisms has exposed the complexity and multiplicity of cellular responses on death receptor activation. It is not surprising, therefore, that understanding the biological function the death receptors lags behind their characterization at the molecular level. In particular, the role of death receptors in many disease states, such as myocardial disease, remains to be elucidated.[38] This complexity in death-receptor function has constrained their potential for pharmacological manipulation. In most cases it is not sufficient to simply activate a specific death receptor. Manipulation of only one of the multiple responses induced by the receptor is desirable. Currently, no solutions to this challenge have been applied.

The exception to this conundrum may be TRAIL. Injection of recombinant TRAIL has few side effects in animal studies and combination therapies that use TRAIL as a radiation sensitizer show early promise.

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Hypothesis

# A docking model of key components of the DISC complex: death domain superfamily interactions redefined

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**Abstract Apoptosis is mediated by a highly regulated signal transduction cascade that eventually leads to precisely directed cell death. The death-inducing signaling complex (DISC), composed of Fas, FADD, and caspase-8, is an apical signaling complex that mediates receptor-induced apoptosis. We have docked the experimentally determined structures of the Fas and FADD death domains into a model of a partial DISC signaling complex. The arrangement of Fas and FADD was determined using the interaction modes of the two heterodimer crystal structures determined to date, Pelle/Tube and Apaf-1/procaspase-9. The proposed model reveals that both interactions can be accommodated in a single multimeric complex. Importantly, the model is consistent with reported site-directed mutagenesis data indicating residues throughout the domain are critical for function. These results imply that members of the death domain superfamily have the potential for multivalent interactions, offering novel possibilities for regulation of apoptotic signaling.** 

*Key words:* Docking model; Apoptosis; Death domain superfamily; Caspase recruitment domain; Death effector domain; Death domain

## 1. Introduction

Apoptosis, or programmed cell death, is a vital cell lifecycle decision point in multicellular organisms. It is a necessary event in normal embryogenesis and development, maintenance of homeostasis, and immune system function [1]. Consistent with this diversity of function, the regulation and initiation of apoptosis is an intricately regulated process, which is achieved through extensive protein–protein interactions. Both the death-inducing signaling complex (DISC) and the apoptosome contain components that use the death domain (DD), death effector domain (DED), or caspase recruitment domain (CARD) to mediate protein–protein contacts [2,3]. These three protein families have been collectively termed the death domain superfamily. These domains are generally used to activate downstream signaling, often in the form of caspase or kinase activation. Each of the three families binds through homotypic interactions, such that DD–DD, DED–DED, and CARD–CARD interactions are thought to be exclusively formed.

Experimental data regarding the structural basis for DD–DD and CARD–CARD interactions have been obtained through the two heterodimer structures of Pelle/Tube and Apaf-1/procaspase-9 [4,5]. The death domain superfamily proteins are composed of an anti-parallel six helical bundle structure in the Greek key topology, with significant variations in the length and positioning of secondary structure elements [6]. The complex between Apaf-1 and procaspase-9 involves interactions between faces of the protein formed chiefly by helices 2 and 3 and helices 1 and 4, respectively (termed a type I interaction) [4]. In contrast, the complex between Pelle and Tube chiefly involves the loops between helices 1 and 2, 4 and 5, and 5 and 6 (type II interaction) [5]. Thus, the prevailing view is that the DD family and the CARD family use fundamentally different heterodimerization modes.

## 2. Materials and methods

The sequence similarity across the death domain superfamily is low, making it difficult to align the sequences based on the primary structure alone. We therefore decided to attempt to rigorously align the three divergent family members using a two step structure-based sequence alignment method. In the first step, each of the three families that comprise the death domain superfamily, DD, DED, and CARD, were iteratively aligned using Psi-BLAST. In the second step, the 10 death domain superfamily members of which the structures are known were superimposed upon $p75^{NTR}$ using DALI [7]. The DALI alignments take into account the tertiary structure, including topological considerations. The DALI superpositions were manually evaluated and optimized, which involved minimizing discrepancies between family members, by calculating the nearest neighbor of each $C_\alpha$ atom of $p75^{NTR}$ to the nine other structures. These alignments form the basis for subsequent superposition calculations.

Superpositions were performed using linear least squares methods as implemented in the program O [8]. The basis of the superpositions was determined from the DALI alignments, in which only the $C_\alpha$ atoms of the homologous residues were used to calculate the rotation and translation matrices. The initial arrangement of the hexamer was determined from a serial superposition of the Apaf-1/procaspase-9 and Pelle/Tube heterodimers. The P1:T1 heterodimer of the two Pelle/Tube heterodimers found in the asymmetric unit cell was chosen. For example, Pelle and Apaf-1 were superimposed, which resulted in the contiguous placement of three death domain superfamily members: Tube, procaspase-9 and the superimposed structures of Pelle plus Apaf-1. Either one of the superimposed structures (Pelle or Apaf-1) is removed, leaving a trimer in which the individual domains interact via type I (Apaf-1/procaspase-9), type II (Pelle/Tube), and the newly formed type III interface. Such superpositions of one member of the heterodimer structures onto a member of the growing multimer are repeated until the heterohexamer is formed. In the final step, Fas

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and FADD were superimposed onto the members of the hexamer (Pelle, Tube, Apaf-1, and procaspase-9).

Interaction interfaces were evaluated based primarily upon electrostatic compatibility. The interaction faces were inspected for electrostatic compatibility by enumerating residues that are 6 Å or less from each other. Precise van der Waals and electrostatic compatibility was not a primary consideration since the interfaces were not optimized using energy minimization to avoid model bias. Plasticity of the interfaces has been experimentally shown in the Pelle/Tube heterodimer in which the two heterodimers in the asymmetric unit have a 7° rotation due to the insertion of a crystallographic related molecule in the interface [5].

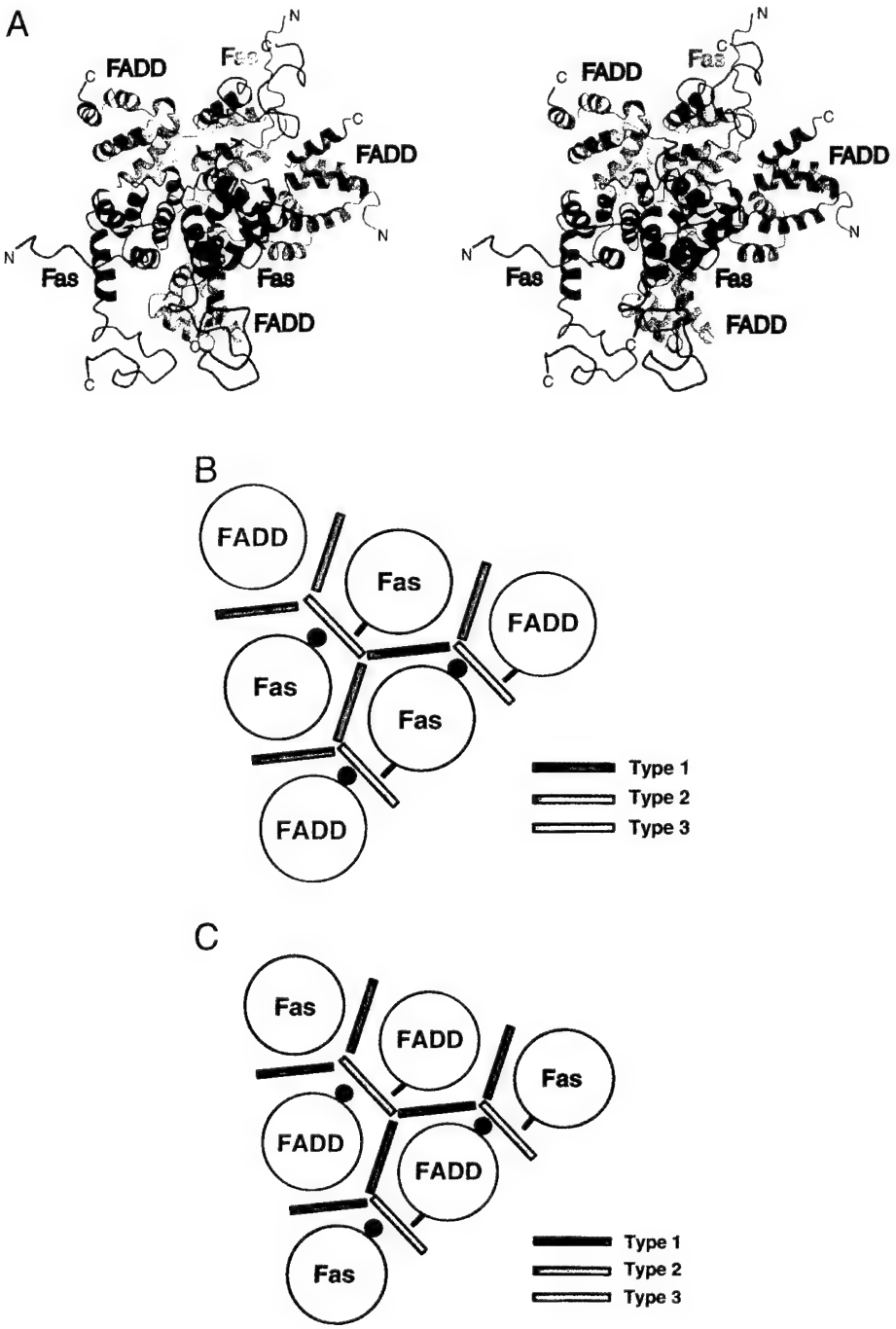


Fig. 1. Docking model of the Fas/FADD DD heterohexamer. A: Ribbon diagram illustrating the three-dimensional relationship found in the heterohexamer composed of Fas (blue) and FADD (red). The view is parallel to and facing the cell membrane. This figure was drawn using Molscript [28] and rendered using POV-Ray. B: Illustration of the three different types of interactions found in a Fas centered DISC complex viewed from the same vantage point as above. The type I interaction (magenta) corresponds to the dimer interface of the CARD of Apaf-1 and procaspase-9 [4]. The type II interface (green) corresponds to the dimer interface of the DD of Pelle and Tube [5]. The type III interaction, shown in cyan, is a novel interaction face formed as two DDs interact via the type I and II interfaces. Thus, this interface is the result of the juxtaposition of a type I and a type II interface. C: Illustration of the FADD centered DISC complex. The vantage point and color coding are identical to that in B.

### 3. Results and discussion

Closer examination of these death domain superfamily structures led us to the striking observation that the type I and type II interactions are not mutually exclusive and, furthermore, superposition of Pelle and procaspase-9 results in a well packed trimer consisting of the DD of Tube, Pelle (or procaspase-9), and Apaf-1. The binding interfaces are adjacent to each other, and form a third interface (type III). These observations, obtained only through rotation and translation of existing structures, prompted this docking model study of Fas and FADD.

Fas, the receptor for Fas ligand, and FADD, an adapter molecule which links Fas to procaspase-8, interact through their DDs [9–11]. The structures of both the Fas DD and the FADD DD have been determined by nuclear magnetic resonance (NMR) [6,12]. To generate a model of the DISC, we have superimposed the DDs of Fas and FADD over the DD of Pelle and Tube, as well as the CARD of Apaf-1 and procaspase-9 using translation and rotation matrices determined from a DALI structural alignment [7]. We extended the docking model from a trimer to a hexamer to accommodate data suggesting that Fas receptor exists as a trimer and each Fas DD binds one FADD DD [13–15]. The additional molecules were positioned using the same rotation matrices calculated to form the trimer complex, thus minimizing manual intervention in the docking process [8]. The resulting model is based completely upon NMR and crystallographic data using known domain–domain interactions to dock Fas and FADD. The proposed complex is structurally and biologically sensible (Fig. 1A).

The C-terminal tails of the DDs, notably of Fas and Tube, were omitted from the sequence alignment due to a lack of significant similarity. In addition, the C-terminal tail of Fas is disordered in the NMR structure [6]. However, analogous to the C-terminal tail of Tube it is likely that the tail of Fas would interact with its partner. Nevertheless, the packing of the C-terminal tail of Tube against Pelle is unlikely to be conserved in detail in the Fas and FADD oligomer. Thus it is not possible to interpret experiments, such as those that implicate the C-terminal tail in lowering the affinity of Fas for FADD [11,16], in the context of the proposed heterohexamer.

The complex consists of six DD modules bound together. The three interfaces form a repeating pattern, reminiscent of a triskelion (Fig. 1). The triskelion occurs three times in the heterohexamer and the identity of the three proteins varies among each, such that each of the three Fas and the three FADD molecules is bound in distinct environments. Each Fas and FADD molecule makes both homo- and heterodimer interactions using each of the three interfaces (Fig. 1B). The

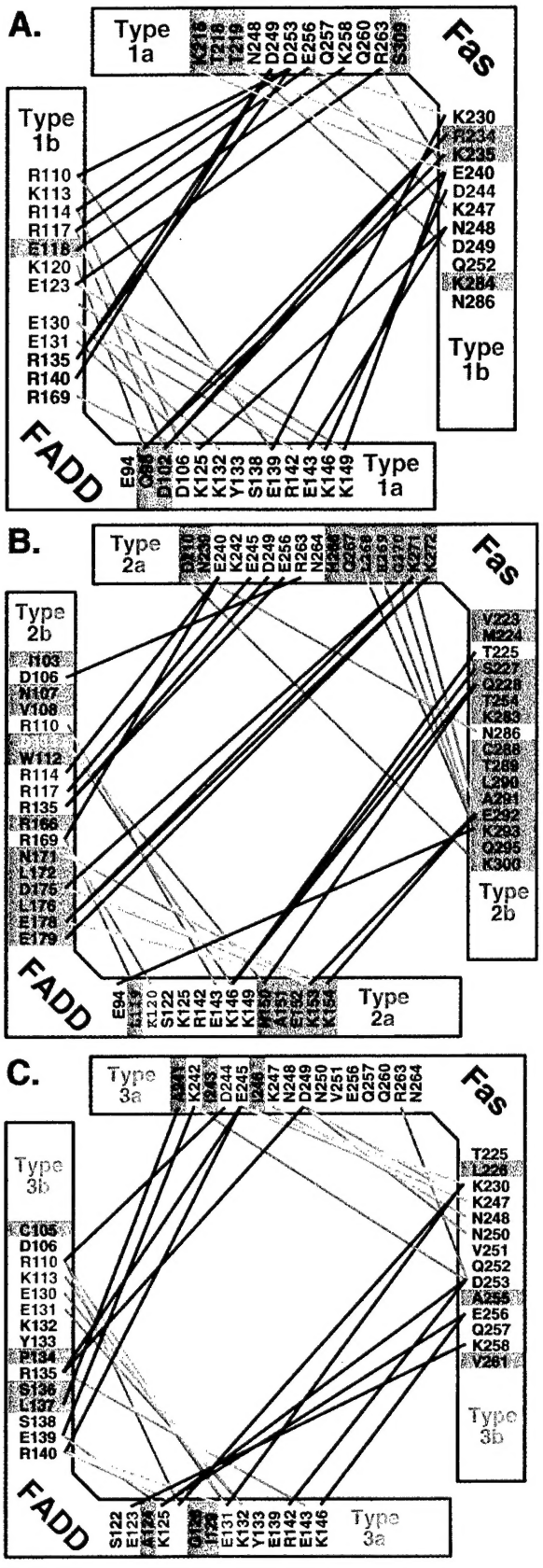


Fig. 2. Diagram indicating residues of Fas and FADD with the potential to interact. Residues within 6 Å of a neighboring molecule (which allows for side chain movement) are indicated. Cyan lines indicate possible homotypic interactions while black lines indicate possible heterotypic interactions. Generally these possible interactions are electrostatic in nature, although selected van der Waals interactions are also indicated. Residues shaded gray are found at a single interaction face only. Residues highlighted red are mutations that eliminate binding or signaling, residues in yellow have a moderate effect, and green residues do not have any effect.
A: Type I interface. B: Type II interface. C: Type III interface.

binding surfaces of Fas and FADD are complementary in both shape and electrostatic nature facilitating the formation of these varied interactions through each of the three interfaces. The interfaces are composed of a mixture of possible van der Waals interactions, hydrogen bonds, and 4–9 salt bridges. Since the interaction surfaces have not been energy minimized to avoid model bias, residues with side chains within 6 Å of each other have been listed in Fig. 2. Several of these residues are specific to a single interface, however many of the amino acids listed can potentially participate in more than one type of interaction. Resolution of these ambiguities will have to await experimental verification to establish any conformational changes and induced fit. Plasticity of the interfaces has been experimentally shown in the Pelle/Tube heterodimer structure where the two dimer interfaces in the asymmetric unit differ by a 7° rotation [5]. The Fas and FADD DDs fit well together, requiring only one loop, which lies between helices 3 and 4, to move. Indeed there is precedent for this movement in the Pelle/Tube heterodimer structure in which this loop of Tube is swung out (Fig. 3) [5].

The proposed docking model is specific and excludes several interactions not observed experimentally. We have attempted to incorporate the solution structure of the FADD DED into the complex [17]. None of the six possible interfaces of either the Fas DD or FADD DD are compatible with those of FADD DED. FADD DED does not form chemically sensible contacts with any of the postulated interfaces of either FADD DD or Fas DD. The interfaces are not electrostatically complementary, resulting in significant charge–charge repulsions (unpublished data). Likewise, a heterohexamer involving either Pelle or Tube and either Apaf-1 or procaspase-9 does not result in a chemically sensible complex, as expected from biological and functional data. This underscores the uniqueness of the FADD/Fas complex and illustrates the specificity of all three modeled interfaces.

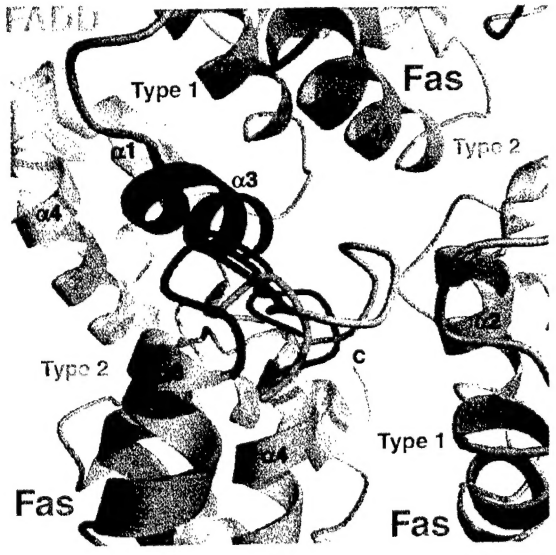


Fig. 3. The loop between helices 3 and 4 at the type III interface is mobile. This loop is displaced in Tube (cyan) relative to FADD (red), Fas (dark blue), and procaspase-9 (green) which prevents steric hindrance between this loop and helix 3 of the neighboring molecule. Three Fas DDs (light blue) and a FADD DD (red) are rendered in ribbon format. The key components of the type III interface, helix 3 of one Fas DD and the loop between helices 3 and 4 of a second Fas DD, are shown in dark blue.

Two fundamentally different heterohexamers can be envisioned, one in which there is a Fas trimer at the center, surrounded by three FADD molecules, and a second, in which a FADD trimer is located at the center surrounded by three Fas molecules (Fig. 1B) [18]. Each of these models is equally probable when based upon a qualitative inspection of the interaction interfaces. The C-termini in the crystal structure of the extracellular domain of tumor necrosis factor receptor (TNFR) bound to TNFβ are relatively far apart (33 Å) [19], providing circumstantial evidence that the three FADD may be located at the center of the heterohexamer. Although the absolute stoichiometry of the members of the DISC complex has not been established, the current model is consistent with a Fas trimer interacting with three FADD molecules. Due to the degenerate nature of the interactions, the formation of larger complexes consisting of multiple trimers cannot be ruled out, although such larger aggregates have not been experimentally observed.

Published site-directed mutagenesis (SDM) data are consistent with the proposed docking model of the DISC complex (Fig. 4). Extensive SDM studies have been carried out using the DDs of human TRADD [20], TNFR [21], and Fas [22–24]. In each case residues affecting binding, function or both were spread throughout the length of the protein. This result is inconsistent with the proposed interaction model of Fas and FADD, in which an anti-parallel interaction involving only helices 2 and 3 has been proposed [12].

Inactivating mutations were found along the length of the Fas DD. Fas mutations which showed binding effects are located in helices 1 through 6, as well as between helices 1 through 5 [23]. Significantly these mutations would affect all three of the proposed interaction types. The DD of TNFR has also been mutated [21], and residues that lower activity can be found in all six secondary structure elements leading to the suggestion that TNFR uses at least two binding interfaces that are each used in homo- as well as heterodimeric interactions [24]. TRADD DD residues have been mutated sequentially to alanine 3–4 amino acid residues at a time [20]. These mutants were analyzed for binding and induction of apoptosis and inactivating mutations were found the length of the protein, not merely at a single DD interface.

Difficulties exist in interpreting these SDM data. For example, Gly 92 and Gln 93 of Pelle are involved in binding Tube, yet G92W, Q93G and Q93W show normal Tube binding activity [5]. Likewise, the TNFR mutants E390A and E406A individually show normal cytotoxic activity, yet when the double mutant is made cytotoxic activity is reduced indicating that elimination of a weak interaction does not necessarily result in a loss of function [21]. Thus, negative SDM results must be interpreted with care. In addition, residues that appear to affect binding or signaling must be regarded with care due to folding and stability effects. For example, the *lpr* mutation in Fas (V238N) results in the local unfolding of helix 3 thereby affecting a significant number of residues beyond Val 238 [25]. Despite these concerns, SDM remains a powerful tool for the analysis of binding interfaces.

Significantly, all of the mutations that show either a full or partial effect are located near one or more of the interfaces. Due to variations in loop lengths and helix positioning a precise correlation between sequence alignments between

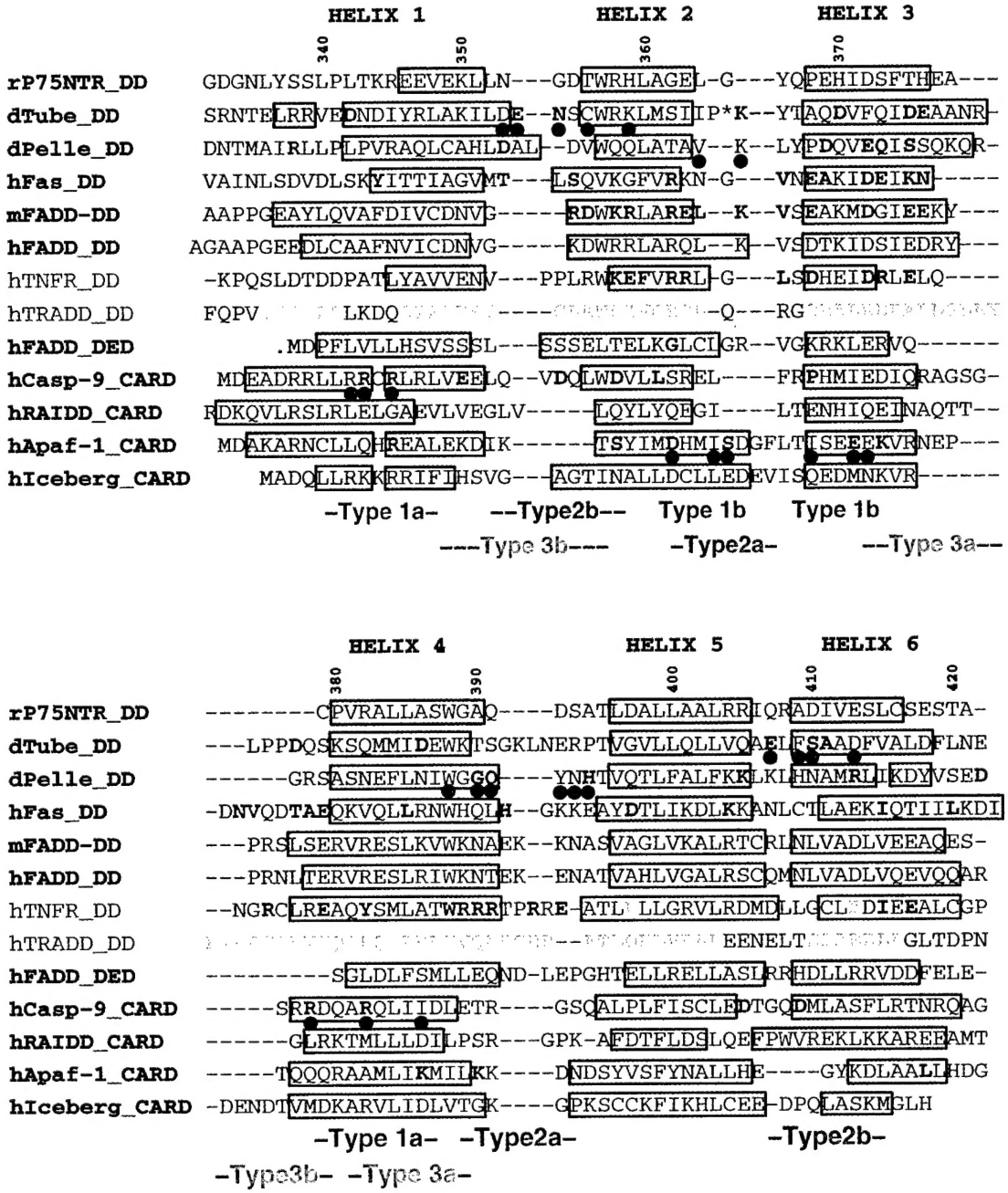


Fig. 4. Structure-based sequence alignment of the death domain superfamily. Residues shown by X-ray crystallography to be involved in heterodimerization are indicated by magenta circles below the sequence [4,5]. Residues highlighted in red have been shown by SDM to be involved in binding or signaling, those shown in yellow show an effect when more than one residue is mutated at a time, those indicated in green show no effect. Blue boxes indicate helices, while proteins named in bold have been structurally characterized. The secondary structure of TNFR has been assigned using NMR, however a complete structure is unavailable at this time [24]. The green asterisk indicates a two helix insert, omitted for clarity, found only in Tube. The C-terminal tail has been omitted due to the low level of sequence homology, although a large number of death domain superfamily members possess a C-terminal extension, including Fas, FADD, and DAP kinase. Structural and mutational references: $p75^{NTR}$ [29], Pelle and Tube [5], Fas [6], FADD [12,17], TNFR [24], TRADD [20], caspase-9 and Apaf-1 [4], RAIDD [30], and Iceberg [31].

proteins with unknown structures and the experimentally determined heterodimer structures is not expected. The significance of the accumulated SDM data lies in the extended pattern of affected residues observed in TNFR, Fas, and TRADD, affecting the length of the DD.

The docking model is testable using targeted SDM initially, culminating in the crystallographic structure determination of the complex. Critical to a SDM analysis, several residues are predicted to make interactions specific to one interface only, especially in the type I and II interfaces, permitting targeted investigation (Fig. 2). Specifically, Lys 215, which is postulated to interact with Glu 240 in a type I Fas–Fas DD interaction, is unique and can be studied (Fig. 2A). The K215E and E240K mutants can be studied for both individual reduction in binding as well as in complementation studies. The type I interface specific to heterodimer formation can be in-

vestigated through mutations of Asp 102 of FADD, which is postulated to interact with either Arg 134 or Lys 235 of Fas, both of which are only involved in the type I interaction. The other two interfaces can be studied through analogous mutations (Fig. 2). An exception is the narrow type III interface consisting mainly of helix 3, which is isolated from the majority of the six helix bundle. Residues of this interface therefore are difficult to unambiguously identify at this point, with the exception of selected hydrophobic residues. It is unclear whether conformational changes, either transmitted from the extracellular domain of Fas or due to DD binding, alter the character of this binding surface. Flexibility in helix 3 has been noted in several NMR studies of DDs [12,24].

The docking model is not only consistent with published SDM data, but also explains diverse phenomena. This hypothesis is needed due to the SDM evidence suggesting that multivalent interactions are common in the death domain superfamily. Tight association of the three Fas molecules may facilitate the transmission of conformational changes from the extracellular portion of the receptor that binds Fas ligand. It has recently been shown that the Fas trimer exists in the absence of Fas ligand binding [15]. This implies that conformational changes must be transferred upon ligand binding. Indeed, it has been shown that a conformational change occurs upon Fas activation using chemical crosslinking experiments [15].

Death effector filaments (DEFs), which are formed by some death domain superfamily members, are extensive, elongated aggregates observed upon overexpression [26]. The formation of DEFs implies that the death domain superfamily forms multivalent and simultaneous interactions consistent with our model. DEFs may form through the preferential binding of monomers to a particular surface, thus forming elongated fibers.

Heterohexamer structures have not been observed in crystallographic analyses so far. However, several of the studied molecules have been altered to optimize solubility [17]. These changes required to obtain heterodimers and avoid aggregation may also have inadvertently eliminated multivalent interaction modes. The C-terminal tail may be indispensable for complex formation as indicated by its pronounced involvement in the type II interface [5]. In addition, full length molecules may be required for complex formation. Full length FADD, containing both the DD and DED, has been shown to self associate by yeast two hybrid studies, yet the isolated FADD DD does not [10,27].

This docking model opens new avenues of study regarding potential interactions between members of the apoptotic signaling machinery. Further Fas and FADD site-directed mutants will have to be generated and quantitative binding studies will have to be performed in order to further substantiate this model. We believe this docking model extends the current monovalent models of death domain superfamily interactions and is more consistent with existing SDM data. It will be interesting to investigate other large signaling complexes such as the apoptosome to determine if similar binding patterns are found.

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